ELECTROPHYSIOLOGICAL INVESTIGATIONS INTO
THE G-PROTEIN MODULATION OF THE NEURONAL
L-TYPE ($\alpha_{1D}$, Cav1.3) AND N-TYPE ($\alpha_{1B}$, Cav2.2)
VOLTAGE DEPENDENT CALCIUM CHANNELS

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of the requirements for the degree of

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'During the last few weeks I’ve been trying to think of something absolutely original and devastating. I’ve been trying to lay my hands on some idea that’ll revolutionise the world in some way. Something like fire …’

Peter Cook

For Mum and Dad,

Thank you for giving me the potential to at least attempt to find something like fire. All I need do now is find the match … and work out which end to strike.
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Abstract

In this thesis two aspects of the G-protein modulation of voltage-dependent calcium channels were investigated using the whole-cell and perforated patch clamp techniques.

1). Neuroendocrine L-type calcium currents play a prominent role in neurosecretion, a process that can be modulated by G-proteins. This G-protein modulatory pathway was investigated using two clonal cell lines: a GH4C1 (derived from rat pituitary tumour tissue) cell line which express predominantly L-type currents; and, an HEK 293 cell line stably expressing a neuronal L-type calcium channel subunit complex (α1D, α2δ, β3). The channel biophysics and pharmacology exhibited by each cell line was shown to be typical of L-type currents. For example, currents in each cell line were shown to be long lasting (displaying little time dependent inactivation) and sensitive to the L-type specific dihydropyridine compounds (e.g. antagonised by nifedipine, and enhanced by the agonist S(-)-BayK8644). Using G-protein coupled receptors expressed in these cells (e.g. endogenous somatostatin type 2 or exogenously expressed dopamine D2 receptors) the G-protein modulation of the L-type calcium currents was investigated by the perfusion of the respective receptor agonists. No G-protein modulation was observed. A more direct method of G-protein activation was attempted, employing the use of the non-hydrolysable GTP and GDP analogues (GTP-γS and GDP-βS): again, no modulation was observed. In contrast, a positive control of HEK 293 cells transfected with α1β, α2δ and β3a (a calcium channel composition known to be G-protein modulated) displayed obvious G-protein modulation in both of these G-protein activating conditions.

2). The role of auxiliary calcium channel β subunits in the G-protein pathway was investigated by transfecting α1B alone or α1B co-expressed with β2a channel subunits in COS-7 cells. Cells expressing α1B/β2a subunits displayed typical G-protein modulation; however, in cells expressing α1B alone G-protein modulation was less apparent and atypical.
### Abbreviations and symbols

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>I-II loop</td>
<td>intracellular loop connecting α1 domains I and II</td>
</tr>
<tr>
<td>7TM</td>
<td>seven transmembrane</td>
</tr>
<tr>
<td>α-AgaIVA</td>
<td>α-Agatoxin fraction IVA</td>
</tr>
<tr>
<td>AID</td>
<td>α-interaction domain</td>
</tr>
<tr>
<td>AKAP</td>
<td>A-kinase anchoring protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>cADPR</td>
<td>cyclic ADP-ribose</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>adenosine triphosphatase</td>
</tr>
<tr>
<td>βARK</td>
<td>β-adrenergic receptor kinase</td>
</tr>
<tr>
<td>BCS</td>
<td>bovine calf serum</td>
</tr>
<tr>
<td>BID</td>
<td>β-interaction domain</td>
</tr>
<tr>
<td>BZA</td>
<td>benzothiazepines</td>
</tr>
<tr>
<td>[Ca²⁺]ᵢ</td>
<td>intracellular free calcium concentration</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>Carb</td>
<td>carbachol</td>
</tr>
<tr>
<td>Cav</td>
<td>VDCC family (revised nomenclature)</td>
</tr>
<tr>
<td>Cᵢ</td>
<td>feedback capacitance</td>
</tr>
<tr>
<td>α-CgTx GVIA</td>
<td>α- Conus geographus Conotoxin GVIA</td>
</tr>
<tr>
<td>α-CmTx MVIIC</td>
<td>α- Conus magus Conotoxin MVIIC</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>Cₘ</td>
<td>whole-cell capacitance</td>
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<tr>
<td>Cᵢ</td>
<td>pipette capacitance</td>
</tr>
<tr>
<td>CICR</td>
<td>calcium-induced calcium release</td>
</tr>
<tr>
<td>CTX</td>
<td>cholera toxin</td>
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<tr>
<td>huD₂ₛ</td>
<td>human dopamine D2 (short) receptor</td>
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<td>rD₂₁</td>
<td>rat dopamine D2 (long) receptor</td>
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<td>DA</td>
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<tr>
<td>DAG</td>
<td>diacyl glycerol</td>
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<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DHP</td>
<td>1,4-didyropyridine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetra acetic acid</td>
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<td>EGTA</td>
<td>ethyleneglycol-bis(b-aminoethyl) N,N,N',N' tetraacetate</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>Forsk</td>
<td>forskolin</td>
</tr>
<tr>
<td>FTX</td>
<td>extract of funnel web spider toxin</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GDP-βS</td>
<td>guanosine-5'-0-(2-thiodiphosphate)</td>
</tr>
<tr>
<td>GITC</td>
<td>guanidium iothiocyanate</td>
</tr>
<tr>
<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>G-protein</td>
<td>guanine nucleotide binding protein</td>
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<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
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<td>GTPase</td>
<td>guanosine triphosphatase</td>
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<td>GTP-γs</td>
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<td>HBSS</td>
<td>HEPES buffered saline solution</td>
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<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
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<tr>
<td>HEPES</td>
<td>N-(2 hydroxyethyl) piperazine-N'-(2 ethane sulphonic acid)</td>
</tr>
<tr>
<td>HVA</td>
<td>high voltage activated</td>
</tr>
<tr>
<td>I</td>
<td>current</td>
</tr>
<tr>
<td>Iᵦᵢ</td>
<td>barium current</td>
</tr>
<tr>
<td>Iₛ</td>
<td>calcium current</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>I_{density}</td>
<td>current density</td>
</tr>
<tr>
<td>I_{hold}</td>
<td>holding current</td>
</tr>
<tr>
<td>I_{max}</td>
<td>maximum peak current</td>
</tr>
<tr>
<td>IP_3</td>
<td>1,4,5- inositol trisphosphate</td>
</tr>
<tr>
<td>IV</td>
<td>current-voltage</td>
</tr>
<tr>
<td>G_{max}</td>
<td>maximum slope conductance</td>
</tr>
<tr>
<td>k</td>
<td>slope factor for activation</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>kHz</td>
<td>kilo Heriz</td>
</tr>
<tr>
<td>LVA</td>
<td>low voltage activated</td>
</tr>
<tr>
<td>M4</td>
<td>muscarinic type 4 receptor</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NIC</td>
<td>nicardipine</td>
</tr>
<tr>
<td>NIF</td>
<td>nifedipine</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PAA</td>
<td>phenylalkylamines</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PKG</td>
<td>protein kinase G</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>+PP</td>
<td>current evoked by a test pulse preceded by large depolarising pre-pulse</td>
</tr>
<tr>
<td>noPP</td>
<td>current evoked by a test pulse without a preceding large depolarising pre-pulse</td>
</tr>
<tr>
<td>Quin</td>
<td>quinpirole</td>
</tr>
<tr>
<td>R_{access}</td>
<td>access resistance</td>
</tr>
<tr>
<td>R_{cell}</td>
<td>input resistance of cell</td>
</tr>
<tr>
<td>R_f</td>
<td>feedback resistance</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>R_s</td>
<td>series resistance</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>RyR</td>
<td>ryanodine receptor</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SNAP-25</td>
<td>synaptosome-associated protein of 25 kDa</td>
</tr>
<tr>
<td>SNARE</td>
<td>soluble NSF attachment protein receptor</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SS-14</td>
<td>somatostatin-14</td>
</tr>
<tr>
<td>syn2</td>
<td>somatostatin type 2 receptor</td>
</tr>
<tr>
<td>synprint</td>
<td>synaptic protein interaction site on the α, VDCC subunit</td>
</tr>
<tr>
<td></td>
<td>time constant of activation</td>
</tr>
<tr>
<td></td>
<td>time constant of inactivation</td>
</tr>
<tr>
<td>TAE</td>
<td>tris-buffered acetate</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline</td>
</tr>
<tr>
<td>TEA</td>
<td>tetraethylammonium salt</td>
</tr>
<tr>
<td>ttp90%</td>
<td>time to 90% of the peak current</td>
</tr>
<tr>
<td>TTX</td>
<td>tetrodotoxin</td>
</tr>
<tr>
<td>V_{50(activ)}</td>
<td>voltage of the mid-point of activation</td>
</tr>
<tr>
<td>V_{50(inact)}</td>
<td>voltage of the mid-point of inactivation</td>
</tr>
<tr>
<td>VDCC</td>
<td>voltage-dependent calcium channel</td>
</tr>
<tr>
<td>V_{hold}</td>
<td>membrane holding potential voltage</td>
</tr>
<tr>
<td>V_{ref}</td>
<td>signal input voltage</td>
</tr>
<tr>
<td>V_{rev}</td>
<td>reversal potential</td>
</tr>
<tr>
<td>V_i</td>
<td>test pulse voltage</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1 Historical background to neurophysiology

1.1.1 The ionic hypothesis

Over six decades ago the modern theories of ion channel biophysics were being formulated in a series of seminal papers. Due to the large size of the giant squid axon (Young, 1936) it became a practical and very fruitful preparation for the early experiments on action potentials. The action potential in the squid giant axon was shown by Cole and Curtis (1939) to be caused by changes in the ion conductance across the axon membrane. This paper also agreed with the dogma that the action potential was due to a loss of the ion selectivity of the membrane, resulting in a loss of the resting membrane potential (Bernstein, 1912; Lillie, 1923). However, this commonly held belief, which only permitted the membrane potential to rise at most to zero, was strikingly opposed by experiments performed by Hodgkin and Huxley (1939). Using an internal electrode within the giant axon, Hodgkin and Huxley showed that the membrane potential reversed from the resting potential of -60 mV to a peak of +40 mV, surpassing the maximal 0 mV potential predicted by Bernstein (1912). A decade later, delayed due to the Second World War, the effects of Na\(^+\) ions flowing through the axon membrane were shown to be responsible for the reversal of the resting potential observed during the action potential (Hodgkin & Katz, 1949). Specific permeation of Na\(^+\) ions down the concentration gradient (the internal axoplasm concentration of Na\(^+\) was shown to be about a tenth of the external solution) accounted for the positive overshoot of the membrane potential. The ionic hypothesis by which the action potential was described by specific conductances of ions, was elegantly described by five papers written by Hodgkin, Huxley and Katz (Hodgkin & Huxley, 1952a,b,c,d; Hodgkin et al., 1952). These papers provided a powerful set of predictive equations quantitatively describing the resting potential and action potentials in terms of specific Na\(^+\) and K\(^+\) conductances. The exacting nature of these equations provided a strong and enduring foundation for modern day ion channel
biophysics. For example, though aqueous pore channels were not initially described in the Hodgkin and Huxley papers, charged particles moving in response to changes in the membrane potential and controlling the ion flux (what we now know to be voltage sensors, gating particles and the channel pore) were predicted, including the number of charged particles (four) that the data in these papers predicted. The description of ‘channels’ and the single-file nature of $K^+$ ion flux were discussed only a few years after the 1952 Hodgkin and Huxley papers (Hodgkin & Keynes, 1955). Considering the timescales involved and the pace of change in modern day science, the foresight provided by the calculations in the Hodgkin, Huxley and Katz papers (Hodgkin & Huxley, 1952a,b,c,d; Hodgkin et al., 1952) is amazing, and recently led to the following commentary by Albright et al. (2000):

The ionic hypothesis of Hodgkin, Huxley and Katz remains one of the deepest insights in neural science. It accomplished for the cell biology of neurons what the structure of DNA did for the rest of biology...It provided a common framework for all electrically excitable membranes and thereby provided the first link between neurobiology and other fields of cell biology.

1.1.2 Early VDCC recordings

It soon became apparent that $Ca^{2+}$ was also an important ion in the mediation of electrical excitability. Experiments by Fatt and Katz (1953) and Fatt and Ginsborg (1958) in crustacean preparations displayed the first currents that were attributable to $Ca^{2+}$ current through voltage-dependent calcium channels. The currents were shown to increase in amplitude with increasing extracellular $Ca^{2+}$, $Ba^{2+}$ and $Sr^{2+}$. In addition, the extracellular application of the quaternary ammonium ion, tetraethylammonium (TEA), was shown to increase the divalent current action potential. In a series of experiments performed by Hille in the 1960-70s (reviewed in Hille et al., 1999), it became apparent that the action of TEA in the studies on crustacean preparations increased the VDCC current by blocking the repolarising $K^+$ channels, allowing the $Ca^{2+}$ influx to self-propagate an action potential. The studies by Hille allowed isolation of $Na^+$ and $K^+$ currents by the specific
block of one ionic current, and provided early evidence for the concept of individual particles or channels for each of the ionic conductances.

1.1.3 Development of patch-clamp electrophysiology

With the early foundations laid for ion channel biophysics, further key developments and evidence for these channel theories were aided by technical developments in the techniques for recording ion currents. In 1976 Neher and Sakmann (1976) described a recording technique in which a patch of cell membrane could be electrically isolated using a recording electrode surrounded by a glass pipette which formed a high resistance seal with the membrane, allowing the recording of tiny single channel ion currents. This patch clamp electrophysiological technique provided a truly molecular definition and biophysical analysis of ion specific channels, confirming the channel specificity suggested initially by Hodgkin and Huxley and subsequently investigated by the channel blocking experiments performed by Hille. Additional refinements by Neher, Sakmann and colleagues (Hamill et al., 1981) in this recording technique (e.g. altering the glass pipette shape design and developing a pipette holder which allowed a negative pressure to be applied to a membrane on which the pipette was applied) provided very stable electrical and mechanical seals between pipette and cell membrane. The resulting gigaohm seals could be used for a number of different recording arrangements (see Hamill et al., 1981) that are routinely used today in ion channel recordings, such as whole-cell patch clamp and inside-out and outside-out patch clamp recordings. The recording solution could be controlled on both sides of the membrane using these gigaohm seal recording techniques allowing greater control of the recording environment in which ion channel currents were recorded.

However, a side effect resulting from the removal of the intracellular environment and essential proteins, so called ‘wash-out’ effects, often resulted in the amplitudes of ion channel currents decreasing rapidly, also known as current ‘run-down’. To reduce these unwanted effects an alternative form of whole-cell patch clamping was
invented: perforated-patch clamp recordings used the pore forming antibiotics nystatin or amphotericin-B in the pipette solution (Horn and Marty, 1988; and, Rae et al., 1991, respectively), creating small pores in the cell membrane. These pores allowed monovalent ions to permeate between the cell and the pipette, providing sufficient electrical access required for voltage clamping of the cell membrane, whilst preventing the loss from the cell of larger multivalent ions and nonelectrolytes, such as proteins, essential to maintain the channel currents.

1.2 Intracellular calcium homeostasis

One of the most important and well-documented effects of the action potential arrival at the pre-synaptic membrane is the membrane depolarisation and gating of VDCCs (Katz & Miledi, 1967; Llinás, 1982). The resulting influx of Ca$^{2+}$ causes the quantal release of neurotransmitters (Fatt & Katz, 1952; del Castillo & Katz, 1954) by the synaptic vesicle exocytotic machinery (see reviews by Sudhof, 1995; Fossier et al., 1999; and Meir et al., 1999) into the pre-synaptic cleft and onto the post-synaptic cell membrane. This complex cascade of protein interactions is carefully choreographed by increases in the concentration of free intracellular cytoplasmic Ca$^{2+}$ ([Ca$^{2+}$]$_i$), and equally important is the rapid and controlled removal of Ca$^{2+}$ returning the [Ca$^{2+}$]$_i$ to resting levels. In addition to Ca$^{2+}$ induced neurotransmitter release, intracellular Ca$^{2+}$ has many other crucial roles, acting as a second messenger within the cell, such as: phosphorylation and dephosphorylation of proteins (and hence long-term potentiation and depression; see review by Milner et al., 1998); excitation-contraction coupling in muscle cells (Tanabe et al., 1988; see also a review by Caterall, 1991); gene transcription (Hardingham et al., 1997; Carrion et al., 1999); and cell apoptosis and necrosis (e.g. during ischaemia Manev et al., 1989; see review by Castillo, 2000).

Thus, the dynamic balance between resting [Ca$^{2+}$]$_i$ and the rapid increase in [Ca$^{2+}$]$_i$ following VDCC activation is of vital importance to the efficient function of the cellular processes. Resting [Ca$^{2+}$]$_i$ is maintained at very low concentrations (~100 nM)
relative to the extracellular $\text{Ca}^{2+}$ concentration, and at highly localised regions of $\text{Ca}^{2+}$ entry and/or release, regions dubbed $\text{Ca}^{2+}$ microdomains, it can rise to >100 $\mu$M (Fogelson & Zucker, 1985; Simon & Llinás, 1985).

### 1.2.1 Increasing [Ca$^{2+}$]$_i$

There are two major routes of increasing [Ca$^{2+}$]$_i$, available to the cell: $\text{Ca}^{2+}$ entry from extracellular sources and $\text{Ca}^{2+}$ release from intracellular stores.

**External $\text{Ca}^{2+}$ entry**

Since the levels of extracellular $\text{Ca}^{2+}$ are maintained at several orders of magnitude greater than [Ca$^{2+}$]$_i$ (several mM extracellularly compared to ~100 nM intracellularly) the gating of $\text{Ca}^{2+}$ permeable channels in the cell membrane allows rapid and large numbers of $\text{Ca}^{2+}$ to enter the cell. The two major types of $\text{Ca}^{2+}$ permeable membrane channels are the VDCC (described in detail later, see section 1.3) and the ligand-gated receptor-operated ion channels (see review by Berridge, 1996). The latter type of calcium channels require the binding of specific ligands to the receptor, causing the associated channel to open, allowing $\text{Ca}^{2+}$ to enter the cell. For example, glutamate binding to NMDA glutamate receptors (Sommer & Seeburg, 1992) or binding to certain AMPA receptor subtypes (see review by Ozawa et al., 1998), or acetylcholine binding to nicotinic acetylcholine receptors, will gate the receptor channels and allow $\text{Ca}^{2+}$ to enter down its electrochemical gradient.

**$\text{Ca}^{2+}$ release from intracellular stores**

Intracellular organelles also provide calcium storage and release mechanisms that contribute to the dynamic changes of [Ca$^{2+}$]$_i$. The endoplasmic reticulum (ER; or sarcoplasmic reticulum in muscle) maintains high (~ mM) calcium concentrations within the ER lumen by a combination of Ca-ATPase pumps at the ER membrane and by $\text{Ca}^{2+}$-binding proteins within the lumen (Meldolesi et al., 1988). This ER store of $\text{Ca}^{2+}$ can be released into the cytosol to increase [Ca$^{2+}$]$_i$. In the membrane of the ER are two $\text{Ca}^{2+}$
release channels: the ryanodine receptor (RyR; see also section 1.4.5) and the inositol 1,4,5-trisphosphate (IP$_3$) receptors (see review by Brini and Carafoli, 2000). The RyR is activated in response to rises in cytosolic [Ca$^{2+}$], a process termed Ca$^{2+}$-induced Ca$^{2+}$ release (CICR; Kuba 1994), and can create waves of Ca$^{2+}$ propagation across the cytosol in response to external Ca$^{2+}$ entry (via VDCCs or ligand-gated calcium channels, see above). It has been postulated that the RyR can also release stored Ca$^{2+}$ by cyclic ADP-ribose (cADPR), and additionally may be activated by another Ca$^{2+}$-releasing messenger, nicotinic acid adenine dinucleotide phosphate (see reviews by Petersen and Cancela, 1999; and, Guse, 2000).

The IP$_3$ receptors, like RyRs, are also sensitive to rises in [Ca$^{2+}$], (Bezprozvanny et al., 1991; Finch et al., 1991). Primarily, however, the IP$_3$ receptors are gated by an increase in IP$_3$ (Berridge, 1993), a second messenger produced by the hydrolysis of phosphatidyl inositol 4,5-bisphosphate, by activation of the phospholipase C pathway in response to activation of metabotropic receptors. The IP$_3$ receptors have also been shown to be involved in a Ca$^{2+}$ feedback inhibition pathway of VDCC currents, providing an example of the complex cross-talk that exists between Ca$^{2+}$ homeostatic pathways (Kramer et al., 1991).

Another intracellular organelle that has Ca$^{2+}$ storage and release mechanisms is the mitochondrion. Mitochondria buffer [Ca$^{2+}$], by removing Ca$^{2+}$ from the cell cytosol during periods of raised [Ca$^{2+}$], (several hundred nM), using a calcium uniporter driven by the electrochemical potential that develops across the inner mitochondrial membrane. When [Ca$^{2+}$], falls below the level of Ca$^{2+}$ in the mitochondria a Na/Ca exchanger releases Ca$^{2+}$ back into the cytosol (Friel & Tsien, 1994). A form of CICR was also shown to exist as another Ca$^{2+}$ buffering mechanism used by mitochondria (Rizzuto et al., 1993; David et al., 1998). A further CICR pathway to which the mitochondria respond has been shown to be caused by increasing [Ca$^{2+}$], resulting in increased pH in the mitochondrial lumen (induced by uptake of Ca$^{2+}$ from the cytosol into the mitochondria). The rise in pH opens a non-selective channel, termed a permeability transition pore,
which releases Ca\(^{2+}\) from the mitochondrion into the cytosol (Ichas et al., 1997). In circumstances where the usual Ca\(^{2+}\) homeostatic mechanisms are insufficient or overcome (e.g. injury leading to ischaemia) and [Ca\(^{2+}\)]\(_{i}\) levels abnormally increase and persist, mitochondria act as Ca\(^{2+}\)-sinks, and buffer [Ca\(^{2+}\)]\(_{i}\) by phosphate precipitation of Ca\(^{2+}\) within the mitochondrial lumen (see review by Brini and Carafoli, 2000).

### 1.2.2 Ca\(^{2+}\) extrusion and maintenance of low [Ca\(^{2+}\)]\(_{i}\)

In order to maintain the low levels of [Ca\(^{2+}\)]\(_{i}\) within the cytosol, in the face of the challenges represented by entry of external Ca\(^{2+}\) or Ca\(^{2+}\) release from internal stores, the cell has several Ca\(^{2+}\) extrusion and buffering pathways. In addition to the Ca\(^{2+}\) buffering afforded by Ca\(^{2+}\) uptake mechanisms in the reticular system and mitochondria (see above, section 1.2.1), there are two main Ca\(^{2+}\) extrusion proteins in the cell membrane. Using ATP as the driving force, a Ca-ATPase allows the exchange of one Ca\(^{2+}\) for the entry of one H\(^+\) (Carafoli, 1992). A second Ca\(^{2+}\) extrusion protein located in the cell membrane is a Na/Ca exchanger, using the high external Na\(^+\) concentration to drive the process, removing a single Ca\(^{2+}\) in response to the entry of three Na\(^+\) (Reuter & Porzig, 1995).

In combination with the Ca\(^{2+}\) pumps actively extruding Ca\(^{2+}\) from the cytosol the cell also contains several Ca\(^{2+}\)-binding proteins (e.g. calbindin, calmodulin, calcineurin, calsequestrin, calreticulin and parvalbumin). These Ca\(^{2+}\)-binding proteins will bind and release Ca\(^{2+}\) in response to changing levels of [Ca\(^{2+}\)]\(_{i}\), dependent upon the dissociation constant of Ca\(^{2+}\) for each of the individual proteins, and in this binding-dissociation cycle will provide a passive form of Ca\(^{2+}\) buffering. Further Ca\(^{2+}\) storage (and release) has also been suggested for a number of intracellular structures including: vesicles, the Golgi apparatus, endosomes and lysosomes (see review by Pozzan et al., 1994). The possibility of the nucleus acting as a dynamic regulator of Ca\(^{2+}\), allowing storage and release of Ca\(^{2+}\) like the ER and mitochondria, has been debated for some time, though direct evidence has been elusive. However, a recent study has shown that the nucleus does in fact have the ability to directly influence [Ca\(^{2+}\)]\(_{i}\): Ca\(^{2+}\) uptake into the nucleus was driven by ATP,
and this nuclear Ca\textsuperscript{2+} store could be released by a cADPR sensitive pathway (Khoo et al., 2000).

1.3 Voltage dependent calcium channels (VDCCs)

1.3.1 VDCC characterisation

To date five different types of VDCC current have been defined according to biophysical and pharmacological characteristics. The five VDCC subtypes have been named L, N, P/Q, R and T. The broadest division of the subtypes can be made according to the voltage dependence of activation. Thus, channels that activate at low voltage thresholds (> -70 mV) have been termed the low voltage activated (LVA) and only contain a single member, the T-type currents. The remaining four VDCC subtypes (L, N, P/Q and R) all activate at voltages with thresholds > -40 mV, and were defined as being high voltage activated (HVA). The other main biophysical and pharmacological characteristics that have been used to delineate each of the VDCC subtypes are summarised in Table 1.1, and will now be discussed briefly.

L, N and T subtypes

In an early characterisation Nowycky et al. (1985) observed the presence of three subtypes of VDCC current in chick dorsal root ganglion (DRG) neurons. According to the channel biophysics and pharmacology of these channel subtypes they were dubbed T (due to the rapid inactivation during depolarisation, or transient nature of the channel current), L (because this subtype displayed little inactivation and were long-lasting) and N-type (neither T nor L; and has subsequently been referred to as the neuronal form, due to its extensive expression in neuronal tissues).

The T-type current had been described by Carbone and Lux (1984) in sensory neurons, though the full classification and naming that was subsequently adopted was performed by Tsien and colleagues (Nowycky et al., 1985; Fox et al., 1987a; Fox et al., 1987b). In these early experiments the T-type current was shown to have a single channel
The classification of VDCC currents according to biophysical and pharmacological properties exhibited. The conductance shown is the single channel conductance for each of the current types. The shaded column denotes the low voltage activated (LVA, threshold of activation >-70 mV) T-type current, all the other (unshaded columns) represent the high voltage activated currents (HVA, threshold of activation >-40 mV). The P- and Q-type currents are shown partially separated to show some of the differences between the two (and the relevant references) though these two currents are routinely collectively referred to as the P/Q type current. The following abbreviations were used: DHP, 1,4-dihydropyridines; PAA, phenylalkylamines; BZT, benzothiazepines; w-CgTx GVIA, w-conotoxin fraction GVIA; w-CmTx MVIIC, w-conotoxin fraction MVIIC; and, w-Aga IVA, w-Agatoxin fraction IVA.

<table>
<thead>
<tr>
<th>Classification</th>
<th>T</th>
<th>L</th>
<th>N</th>
<th>P/I</th>
<th>Q</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inactivation rate (ms)</strong></td>
<td>fast</td>
<td>slow</td>
<td>intermediate</td>
<td>slow</td>
<td>intermediate</td>
<td>fast</td>
</tr>
<tr>
<td></td>
<td>(20-50)</td>
<td>(&gt;500)</td>
<td>(50-500)</td>
<td>(&gt;500)</td>
<td>(&gt;100)</td>
<td>(20-30)</td>
</tr>
<tr>
<td><strong>Relative conductance</strong></td>
<td>$\text{Ba}^{2+}=\text{Ca}^{2+}$</td>
<td>$\text{Ba}^{2+}&gt;\text{Ca}^{2+}$</td>
<td>$\text{Ba}^{2+}&gt;\text{Ca}^{2+}$</td>
<td>?</td>
<td>?</td>
<td>$\text{Ba}^{2+}&gt;\text{Ca}^{2+}$</td>
</tr>
<tr>
<td><strong>Conductance (pS)</strong></td>
<td>7-10</td>
<td>9-26</td>
<td>10-20</td>
<td>10-20</td>
<td>10-20</td>
<td>15</td>
</tr>
<tr>
<td><strong>Selective pharmacology</strong></td>
<td>kurtoksin (&lt;350nM)</td>
<td>DHP?</td>
<td>$\omega$-CgTx GVIA (&lt;100nM)</td>
<td>$\omega$-Aga IVA (&lt;100nM)</td>
<td>$\omega$-Aga IVA (&gt;100nM)</td>
<td>?</td>
</tr>
<tr>
<td><strong>References</strong></td>
<td>Nowycky et al., 1985</td>
<td>Nowycky et al., 1985</td>
<td>Nowycky et al., 1985</td>
<td>Linas et al., 1989</td>
<td>Hillyard et al., 1992</td>
<td>Randall et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Fox et al., 1987a,b</td>
<td>Fox et al., 1987a,b</td>
<td>Fox et al., 1987a,b</td>
<td>Usosowicz et al., 1992</td>
<td>Randall et al., 1993</td>
<td>Zhang et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Huguenard, 1996</td>
<td>Kasai et al., 1987</td>
<td>Mintz et al., 1992a,b</td>
<td></td>
<td>Randall and Tsien, 1995</td>
<td></td>
</tr>
</tbody>
</table>
conductance of 8 pS and an increased sensitivity to block by Ni$^{2+}$ compared to Cd$^{2+}$. The search for a selective pharmacological blocker for the T-type current has been largely unsuccessful: mibefradil had been suggested to be selective in the block of T-type currents (Mishra & Hermsmeyer, 1994), but has been shown to block other VDCC current types (Bezprozvanny & Tsien, 1995; Jimenez et al., 1999). The most promising T-type selective agent at present appears to be kurtoxin, a toxin derived from the South African scorpion (*Parabuthus transvaalicus*; Chuang et al., 1998).

L-type VDCC currents are distinguished from the T-type by their increased permeability to Ba$^{2+}$ than to Ca$^{2+}$, an increased sensitivity to block by Cd$^{2+}$ compared to Ni$^{2+}$ and relatively large single channel conductance of 25 pS (Nowycky et al., 1985; Fox et al., 1987a; Fox et al., 1987b). Further study of L-type currents has shown that L-type currents have two conductance states in physiological levels of calcium (Church & Stanley, 1996). In addition the L-type currents were shown to be sensitive to 1,4-didyropyridine (DHP) antagonists (e.g. nifedipine, nimodipine, nicardipine) and agonists (e.g. S(-)-BayK8644). However, the selectivity of the DHPs for L-type currents has been questioned since DHP antagonists (Richard et al., 1991; Stephens et al., 1997; Furukawa et al., 1999) and DHP agonists (Lacinova et al., 2000) have effects upon other VDCC current subtypes (see also section 4.3.2). Other pharmacological agents to which L-type currents have been shown to be sensitive, include the phenylalkylamines (PAA, e.g. verapamil) and benzothiazepines (BZA, e.g. diltiazem)(Lee and Tsien, 1983; for reviews see Fleckenstein, 1983; and Clusin and Anderson, 1999). Once again, however, PAA and BZA block other VDCC current types (Diochot et al., 1995), questioning the L-type current selectivity of these compounds.

The third VDCC current sub-type of the characterisation of currents in DRG neurons by Tsien and colleagues, was the N-type. Like the L-type current, the N-type was a HVA current which displayed an increased current amplitude using Ba$^{2+}$ compared to Ca$^{2+}$ as the charge carrier. The N-type current displayed an inactivation rate and single channel conductance (13 pS) intermediate to that measured for the T- and L-type
currents. The N-type current has subsequently been shown to display a higher single channel conductance (18 pS; Plummer et al., 1989). The key selective agent in defining N-type currents was a fraction of venom from the piscivorous cone snail, Conus geographus. The fraction, α-conotoxin GVIA (α-CgTx GVIA), potently, selectively (<100 nM) and irreversibly blocks the N-type current (Kasai et al., 1987; Olivera et al., 1994).

P/Q and R subtypes

Following the characterisation of the initial trio of VDCC current subtypes a further three subtypes were defined. The P/Q and R-type VDCC currents were named following the L, N and T-type nomenclature adopted by Nowycky et al. (1985).

P-type current were initially classified in Purkinje neurons (Llinas et al., 1989), in which the VDCC current population is predominantly (>80%) P-type in nature. The P-type currents display little current inactivation over long depolarisation steps (1 s) and have been shown to exhibit multiple conductance levels (9, 14 and 19 pS in 100 mM Ba$^{2+}$), which could represent different channels or sub-conductance levels (Usowicz et al., 1992). Initial toxin selectivity was shown using a polyamine fraction of a spider (Agelenopsis aperta) venom, FTX (Llinas et al., 1989). A second fraction of this spider venom, α-Agatoxin IVA (α-AgaIVA), was shown to be more potent and selective (<100 nM) in the block of P-type currents (Mintz et al., 1992a,b).

The Q-type current, initially identified in cerebellar granule cells, was similar in its pharmacological profile to the P-type, and the separation of the currents was debatable, often being grouped and labelled P/Q type currents (evidence now suggests P and Q-type currents are separable, being formed from splice variants of the α_{1A} gene, see Bourinet et al., 1999; Hans et al., 1999; see also section 1.3.3 for further discussion). However, pharmacological and biophysical differences between P and Q type currents that were suggested and defined as follows: the Q-type shows an increased inactivation rate over the depolarising step compared to P-type (Zhang et al., 1993; Randall et al., 1993). The
Q-type current is sensitive to α-AgaIVA, though at concentrations greater than that considered specific to be specific for the P-type current (>100 nM).

After the application of selective toxins and pharmacological agents for the known HVA VDCC current types (L, N, P/Q-types) it was observed in cerebellar granule cells that a small residual or resistant HVA current remained. This resistant fifth current was therefore dubbed the R-type current (Randall et al., 1993; Zhang et al., 1993; Randall and Tsien, 1995). This VDCC current subtype was shown to display rapid inactivation, and was also more sensitive to Ni\(^{2+}\) than Cd\(^{2+}\). However, these small residual currents have been suggested to be remnants of existing VDCC currents, ostensibly appearing to be a current subtype, but possibly arising due to incomplete block of other current subtypes. For instance, it has been suggested that the high Ba\(^{2+}\) used in the study of Ca\(^{2+}\) currents may be causing experimental artefacts such as incomplete toxin block (Albillos et al., 1996).

1.3.2 VDCC molecular structure

During the characterisation of the VDCC current types, the molecular counterpart of each of these current types was also being investigated. However, isolation of VDCC proteins initially proved problematic due to the relatively low densities of protein recoverable from cell membranes. The transverse tubule system in skeletal muscle proved to be the key, providing sufficient quantities of the skeletal muscle DHP-sensitive Ca\(^{2+}\) channel (Glossman et al., 1983). Purification using lectin immobilisation of the skeletal muscle calcium channel, due to it being a glycoprotein, was performed (Curtis & Catterall, 1984; Borsotto et al., 1984) and the subsequent biochemical analysis elucidated the protein structure of the skeletal muscle L-type VDCC, which was used as the archetypal VDCC complex for the subsequent VDCC family members.
VDCC are hetero-oligomeric complexes – studies on the skeletal muscle L-type channels

The purified skeletal muscle L-type VDCC protein was analysed by several laboratories (Hosey et al., 1987; Morton and Froehner, 1987; Takahashi et al.; Tananbe et al., 1987; Vaghy et al., 1987), and was shown to be a pentameric complex comprising five hetero-oligomer subunits. The α₁ subunit was a protein of 175 kDa (labelled α₁5 in this skeletal muscle preparation) and has since been shown to be the main pore-forming subunit, with functional domains for the major channel characteristics (such as the voltage sensor, ion selectivity filter, and channel activation and inactivation; see section 1.3.3 below). The α₁ subunit co-purified with an α₂δ, a β and a γ protein subunit. These protein subunits are viewed as accessory or auxiliary subunits to the main pore-forming α₁ subunit, altering and shaping the channel biophysics (see section 1.3.4 below). The α₂ subunit (with an estimated mass of between 135-150 kDa) was covalently bound to a δ subunit (approximately 24-29 kDa) by a disulphide bridge, effectively behaving as a single protein subunit. The β (52 kDa) and γ (32 kDa) subunits completed the complex forming the skeletal muscle VDCC. A schematic of the arrangement of each of these subunits is shown in Figure 1.1. Following the protein chemistry, purification and subunit determination of the skeletal muscle L-type VDCC, the individual subunit genes were

Figure 1.1 Simple schematic showing the VDCC hetero-oligomer

The pore forming α₁ subunit (unshaded) and the associated auxiliary subunits (α₂δ, β and γ, all shaded grey), determined from the analysis of the purified skeletal muscle L-type VDCC.
cloned from rabbit skeletal muscle: α₁ (Tanabe et al., 1987), α₂δ (Ellis et al., 1988), β (Ruth et al., 1989) and γ (Bosse et al., 1990; Jay et al., 1990).

These initial cloning studies heralded an era starting in the latter part of the 1980s and continuing through to the present day, in which a strong synergy between the cloning and DNA/protein manipulation of molecular biology and electrophysiology provided a rich vein of research in the ion channel field. The cloning of numerous VDCC genes has led to the molecular derivation of most of the VDCC subtypes previously characterised by biophysics and pharmacology (section 1.3.1). Chimaeric and mutated forms of these channel clones have elicited information on the functional domains essential for the inherent channel biophysical properties. It is to this molecular structural definition of VDCCs that this discussion addresses in the following sections (sections 1.3.3 and 1.3.4).

1.3.3 α₁ VDCC subunits

Primary structure of α₁ VDCC subunit indicates a structural topology homologous to voltage-dependent Na⁺ channel

The primary structure of membrane proteins maybe modelled into predictable secondary structural regions, depending on the numbers of hydrophobic and hydrophillic amino acid residues. From such hydrophobicity plots the α₁ subunit was predicted to comprise four repeating domains (I-IV), with each domain consisting of 6 transmembrane α-helices (S1-6), and intracellular amino (N) and carboxyl (C) termini. This overall topology was highly analogous to voltage-dependent Na⁺ (and K⁺) channels (see review by Guy and Conti, 1990). The voltage sensor of the VDCC is formed of positive amino acid residues found on the S4 transmembrane helices on each of the four domains. The VDCC pore and ion selectivity filter has been localised to the linking polypeptide between the S5 and S6 α-helices, again within all four domains. The voltage-sensor and pore (and ion selectivity) α₁ functional domains are discussed later in this section. The predicted
secondary structure is shown later in Figure 1.3, when discussing the molecular
determinants of the channel characteristics.

\[ \alpha_1 \text{ VDCC subunit genes} \]

Following the isolation and cloning of the VDCC subunits of the skeletal muscle L-type
channel, genes for other forms of VDCC subunits were elucidated and cloned by
homology to the original clone. In addition to \( \alpha_{1S} \), the cloning of \( \alpha_1 \) VDCC subunits has
revealed nine further genes (\( \alpha_1 \, \text{A-I} \)), with more variants created by alternative splicing
within the genes. Functional expression has been performed with eight of these \( \alpha_1 \) clones
(\( \alpha_{1F} \) remains to be functionally expressed). The recorded currents arising from the \( \alpha_1 \)
subunit expression, usually in combination with auxiliary subunits, has been
biophysically and pharmacologically defined, providing a molecular derivation for most
of the known VDCC current types (discussed previously in section 1.3.1). Table 1.2
summarises the known \( \alpha_1 \) VDCC subunit genes in relation to their functional current
correlates, and provides gene and tissue localisation for each. The auxiliary subunits are
discussed separately (see section 1.3.4). In the following sections the cloning and
functional expression of the L-type, non-L-type and the low voltage activated VDCC
genes will be discussed.

\[ \text{L-type} \, \alpha_1 \text{ subunit genes} \, (\alpha_{1C}, \alpha_{1D}, \alpha_{1F}) \]

\( \alpha_{1C} \) isoform:

\( \alpha_{1C} \) was the first clone to be isolated following the \( \alpha_{1S} \) derivation. Isolation of the \( \alpha_{1C} \)
protein was performed in a cardiac preparation, and the cDNA was cloned from rabbit
heart cDNA libraries by homology using an \( \alpha_{1S} \) probe (Mikami \textit{et al.}, 1989). Though
often referred to as the cardiac L-type clone, \( \alpha_{1C} \) has been found in a large number of
tissues (Hell \textit{et al.}, 1993). The \( \alpha_{1C} \) gene appears to be alternatively spliced at six points
(Perez-Reyes \textit{et al.}, 1990), though there are probably only three splice variants that are
functionally expressed.
The table shows a summary of the molecular cloning and tissue distribution of the VDCC α₁ subunits, and the consensus opinion of how the expressed α₁ VDCC subunits correlate to the previously classified VDCC currents (see Table 1.1). Where possible the tissue distribution relates to the major areas of protein expression, generally defined by immunostaining. However, the distribution of the more recent isoforms (α₁F, α₁G) was defined by mRNA detection.

<table>
<thead>
<tr>
<th>α₁ Isoform</th>
<th>Functional current</th>
<th>Gene name (HUGO)</th>
<th>Chromosomal localisation (human)</th>
<th>Splice variants</th>
<th>Tissue localisation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P/Q Type</td>
<td>High voltage activated, non L-type</td>
<td>CACNA1A</td>
<td>19p13.1-2</td>
<td>α₁A,b</td>
<td>brain, cochlea, adrenal</td>
<td>Starr et al. (1991); Mori et al. (1991); Sather et al. (1993); Stea et al. (1994); Dinon et al. (1995); Westenbroek et al. (1995); Ophoff et al. (1996); Bourinet et al. (1999)</td>
</tr>
<tr>
<td>N-type</td>
<td>High voltage activated, L-type</td>
<td>CACNA1B</td>
<td>9q34</td>
<td>α₁B.1</td>
<td>brain, nervous system</td>
<td>McEnery et al. (1991); Horne et al. (1991); Dubel et al. (1992); Williams et al. (1992a); Brust et al. (1993); Fujita et al. (1993); Stea et al. (1993); Witcher et al. (1993); Dinon et al. (1995); Chung et al. (2000)</td>
</tr>
<tr>
<td>R-type?</td>
<td>High voltage activated, L-type</td>
<td>CACNA1E</td>
<td>1q25-q31</td>
<td>α₁E</td>
<td>brain, cochlea, retina</td>
<td>Nidome et al. (1992); Ellinor et al. (1993); Horne et al. (1993); Soong et al. (1993); Schneider et al. (1994); Wako et al. (1994); Williams et al. (1994); Dinon et al. (1995); Yoko et al. (1995); Page et al. (1998)</td>
</tr>
<tr>
<td>L-type</td>
<td>Low voltage activated</td>
<td>CACNA1C</td>
<td>12p13.3</td>
<td>α₁C-a</td>
<td>heart</td>
<td>Mikami et al. (1989); Biel et al. (1990); Koch et al. (1990); Snutch et al. (1991); Hell et al. (1993); Schultz et al. (1993)</td>
</tr>
<tr>
<td>CACNA1D</td>
<td>L-type</td>
<td>3p14.3</td>
<td>α₁D-a</td>
<td>brain, pituitary, pancreas, kidney, ovary, cochlea; heart</td>
<td>Perez-Reyes et al. (1990); Hui et al. (1991); Chin et al. (1992); Seino et al. (1992); Williams et al. (1992b); Yaney et al. (1992); Hell et al. (1993); Ihara et al. (1995); Wyatt et al. (1997)</td>
<td></td>
</tr>
<tr>
<td>L-type</td>
<td>Low voltage activated</td>
<td>CACNA1F</td>
<td>Xp11.23</td>
<td>α₁F</td>
<td>retina</td>
<td>Bech-Hansen et al. (1998); Strom et al. (1998)</td>
</tr>
<tr>
<td>L-type?</td>
<td>Low voltage activated</td>
<td>CACNA1S</td>
<td>1q31-q32</td>
<td>α₁S-a</td>
<td>skeletal muscle</td>
<td>Tanabe et al. (1987); Perez-Reyes et al. (1988); Lacerda et al. (1991); Varadi et al. (1991); Gregg et al. (1993); Hogan et al. (1994)</td>
</tr>
<tr>
<td>L-type?</td>
<td>Low voltage activated</td>
<td>CACNA1G</td>
<td>17q22</td>
<td>α₁G</td>
<td>brain, nervous system, pituitary</td>
<td>Perez-Reyes et al. (1998); Klugbauer et al. (1999); Talley et al. (1999)</td>
</tr>
<tr>
<td>L-type</td>
<td>Low voltage activated</td>
<td>CACNA1H</td>
<td>16p13.3</td>
<td>α₁H</td>
<td>heart, brain, kidney, liver, nervous system, pituitary</td>
<td>Cribbs et al. (1998); Talley et al. (1999)</td>
</tr>
<tr>
<td>L-type</td>
<td>Low voltage activated</td>
<td>CACNA11</td>
<td>22q13</td>
<td>α₁1</td>
<td>brain, nervous system, pituitary</td>
<td>Lee et al. (1999); Talley et al. (1999)</td>
</tr>
</tbody>
</table>
Expression of the $\alpha_{1C}$ cRNA in the *Xenopus laevis* oocyte expression system, allowed VDCC currents that were sensitive to DHPs to be recorded (Mikami *et al.*, 1989; Schultz *et al.*, 1993). These and other studies provided evidence that this $\alpha_{1C}$ clone was the molecular correlate of cardiac L-type currents that had been described previously.

Additional co-expression with auxiliary VDCC subunits, such as $\alpha_2\delta$ and $\beta$ subunits, also increased the $\alpha_{1C}$ current amplitude ($\alpha_2\delta$ effect; Mikami *et al.*, 1989), shifted the voltage-dependence of activation to more hyperpolarised potentials and accelerated the rate of activation ($\beta$ subunit effects; Wei *et al.* 1991). The auxiliary subunits will be discussed further in section 1.3.4.

$\alpha_{1D}$ isoform:

Another L-type isoform, $\alpha_{1D}$, was cloned using a combination of $\alpha_{1S}$ cDNA probes, PCR and oligonucleotides designed by comparing conserved sequences in the two ($\alpha_{1S}$ and $\alpha_{1C}$) previously cloned L-type $\alpha_1$ subunits (Perez-Reyes *et al.*, 1990; Hui *et al.*, 1991; Seino *et al.*, 1992; Yaney *et al.*, 1992; Williams *et al.*, 1992b; Nobes *et al.*, 1998). The tissue localisation was shown to be broad (see Table 1.2; Hell *et al.*, 1993; Wyatt *et al.*, 1997), though it was particularly prevalent in neuronal and pituitary tissue, and has therefore been implicated as the pore forming subunit mainly responsible for the neuroendocrine L-type current. The $\alpha_{1D}$ cDNA sequence was shown to be very similar to the $\alpha_{1C}$ clone, displaying 70% identical sequence across all the deduced sequence, and up to 90% homology when comparing the conserved transmembrane and pore sequences (see Figure 1.2, later). Splice variants have been discovered ($\alpha_{1D-a}$), with most variation occurring in the long C-terminus. The splice variant used in the study described here was the human $\alpha_{1D-a}$ (see Chapter 4), and has a full length C-terminus, including numerous potential sites for channel modulation. The $\alpha_{1D-a}$ splice variant is formed of 2161 amino acids, and has 3 of 12 potential N-glycosylation sites, 9 of 12 potential cAMP-dependent phosphorylation and 22 of 26 potential protein kinase C phosphorylation sites (all these
sites, along with the full cDNA and amino acid sequence are shown in Williams et al., 1992b).

Considering the numbers of clones isolated by different groups, the functional characterisation of α₁D VDCC currents has been limited. Williams et al. (1992b), who provided the α₁D-a stably expressing HEK 293 cell line used in the present study, were successful in observing calcium channel currents by coinjecting cRNA for the human α₁D-a with the auxiliary α₂β₃δ-1 and β₂ into Xenopus oocytes. The calcium channel currents recorded from this expression were sensitive to nifedipine and S(-)-BayK8644, and characteristic of L-type channel currents were also HVA and displayed long-lasting, non-inactivating currents. Therefore, this expressed channel was a strong candidate for the neuroendocrine L-type current. Another group also recorded α₁D-a calcium channel currents in Chinese hamster ovary (CHO) cells which had additionally been stably transfected with the β₂ VDCC subunit cDNA (Ihara et al., 1995). However, these currents observed were small (<200 pA after enhancement by S(-)-BayK8644 application), and extensive pharmacology and biophysics of the expressed currents were not performed.

Stable expression of a combination of the human α₁D-a, β₃a and α₂β₃δ-1VDCC subunits in human embryonic kidney (HEK) 293 cells has provided a mammalian expression system with currents that display typical L-type pharmacology and biophysics, though they have only been published in abstract form so far (Hans et al., 1997; Brust et al., 1997). Data recorded from these stably transfected HEK 293 cells, presented here, has been submitted for publication (Bell et al.).

α₁F isoform:

A fourth putative L-type gene, α₁F, has recently been cloned (Strom et al., 1998; Bech-Hansen et al., 1998). The α₁F gene has been shown to be mutated in X-linked congenital stationary night blindness (Strom et al., 1998; Bech-Hansen et al., 1998). As yet, however, functional expression the α₁F gene has not been performed, though by sequence
homology it has been suggested to be an L-type $\alpha_1$ VDCC subunit (see the $\alpha_1$ VDCC subunit phylogenetic tree, Figure 1.2, later).

Non-L-type $\alpha_1$ subunit genes ($\alpha_{1A}, \alpha_{1B}, \alpha_{1E}$)

$\alpha_{1A}$ isoform:

The $\alpha_{1S}$ cDNA was again used as probe to find the $\alpha_{1A}$ sequence in rabbit and rat brain cDNA libraries (Mori et al., 1991; Starr et al., 1991). The functional expression of this VDCC subunit cRNA in Xenopus oocytes did produce VDCC currents, though robust currents were only observed with co-expression of $\alpha_2\delta$-1 and $\beta_1$ (Mori et al., 1991).

Confusion arose over the VDCC current type that was observed when expressing the $\alpha_{1A}$ subunit: abundance of $\alpha_{1A}$ mRNA transcripts in Purkinje neurons, the original neuronal preparation in which P-type currents were defined (Llinas et al., 1989), suggested that $\alpha_{1A}$ may encode the P-type current (Mori et al., 1991). However, expression in Xenopus oocytes of the $\alpha_{1A}$ VDCC subunit cDNA produced currents that were less sensitive to $\omega$-AgIVA, but more sensitive to $\omega$-CmTx MVIIC than the Purkinje neuron P-type current (Sather et al., 1993; Stea et al., 1994). In mammalian COS-7 cells, expression of $\alpha_{1A}$ gave currents with greater sensitivity to $\omega$-AgIVA (Berrow et al., 1997). It appeared that currents arising from $\alpha_{1A}$ expression had qualities characteristic of both the P- and Q-type VDCC currents, and $\alpha_{1A}$ was therefore deemed to be the molecular counterpart of P/Q-type currents (Wheeler et al., 1994). The grouping of the $\alpha_{1A}$ VDCC current into the P/Q-type classification, however, was never sufficient since the $\alpha_{1A}$ derived currents did not accurately reflect either of the characteristics defined for the separate P- and Q-type currents (Randall & Tsien, 1995). An interesting recent development provides evidence for the clarification of the $\alpha_{1A}$ derived VDCC currents. The discovery of two $\alpha_{1A}$ splice variants ($\alpha_{1A,1}$ and $\alpha_{1A,2}$ by Hans et al., 1999; or alternatively labelled $\alpha_{1A,a}$ and $\alpha_{1A,b}$ by Bourinet et al., 1999) may provide sufficient $\alpha_{1A}$ isoform variation to account for both the P- and Q-type currents. Further, Bourinet et al. (1999) have shown that the alternative $\alpha_{1A}$
splice variants have distinctive \(\omega\)-AgaIVA sensitivities and also display different inactivation rates. Such pharmacological and biophysical characteristic differences are key to the definition of the P- and Q-type currents, providing the \(\alpha_{1A}\) gene with sufficient variety to possibly account for both current types.

\(\alpha_{1B}\) isoform:

The N-type VDCC current has a clear molecular counterpart in the \(\alpha_{1B}\) gene product. Further to this observation, the \(\alpha_{1B}\) gene product was isolated and purified from brain by using the N-type specific toxin \(\omega\)-CgTx GVIA as a highly selective marker (McEnery \textit{et al.}, 1991; Witcher \textit{et al.}, 1993). The \(\alpha_{1B}\) protein, like the \(\alpha_{1S}\) channel, was also shown to be complexed with the auxiliary subunits \(\alpha_\delta\) and \(\beta_3\) (Witcher \textit{et al.}, 1993). Expression of \(\alpha_{1B}\), with the auxiliary subunits, in \textit{Xenopus} oocytes (Dubel \textit{et al.}, 1992) and in HEK 293 cells (Williams \textit{et al.}, 1992b) increased the binding of \(^{125}\)I-labeled \(\omega\)-CgTx GVIA; the latter study using HEK 293 cells also observed VDCC currents that were potently blocked specifically by \(\omega\)-CgTx GVIA.

Cloning of the \(\alpha_{1B}\) gene was performed using \textit{in vitro} screening of brain cDNA libraries using \(\alpha_{1S}\) (Dubel \textit{et al.}, 1992; Williams \textit{et al.}, 1992a) or \(\alpha_{1A}\) (Fujita \textit{et al.}, 1993) cDNA as probes. In addition to these mammalian \(\alpha_{1B}\) clones, an \(\alpha_{1B}\) homologue was identified and cloned in the marine ray (\textit{Discopyge ommata}; Horne \textit{et al.}, 1991).

\(\alpha_{1E}\) isoform:

The \(\alpha_{1E}\) gene was another gene to be discovered using \textit{in vitro} cDNA probe searches of rat, rabbit and human brain cDNA libraries (Niidome \textit{et al.}, 1992; Soong \textit{et al.}, 1993; Schneider \textit{et al.}, 1994; Williams \textit{et al.}, 1994). During initial studies on the current arising from \(\alpha_{1E}\) expression, it was thought that the biophysics of the current, displaying a relatively low voltage for activation and rapid activation and inactivation current kinetics, may define the current as a member of the LVA or T-type group of currents (Soong \textit{et al.}, 1993). However, further investigation of the current derived from \(\alpha_{1E}\) expression has
argued against the LVA/T-type current classification; other groups have shown that the
activation and inactivation characteristics are more HVA-like, and the $\alpha_{IE}$ derived current
has a larger single-channel conductance (14 pS) than the LVA T-type current (~8
pS)(Schneider et al., 1994; Williams et al., 1994).

The expression of $\alpha_{IE}$ creates VDCC currents that are largely resistant to the
main pharmacological and toxins that have defined the other VDCC current types (Ellinor
et al., 1993; Soong et al., 1993; Williams et al., 1994). Thus, the lack of sensitivity to
DHPs, phenylalkylamines, $\omega$-CgTx GVIA or funnel web spider toxin fraction FTX, has
led to the suggestion that the $\alpha_{IE}$ gene product may provide the main pore forming
subunit of the R-type currents (Zhang et al., 1993; Randall & Tsien, 1997).

Low voltage activated $\alpha_{I}$ subunit genes ($\alpha_{IG}$, $\alpha_{IH}$, $\alpha_{II}$)

A number of VDCC subunit genes that were cloned using existing subunit sequences as
search sequences for related genes in ever expanding, though largely undefined, gene
sequence databases. These computer based gene sequence searches, comparable to
fishing in cDNA sequence libraries with cDNA probes in vitro, has been dubbed in silico
cloning. To date in silico cloning has unearthed three T-type genes ($\alpha_{IG}$), two additional
$\alpha_{3}\delta$ genes ($\alpha_{3}\delta$-2 and -3) and four putative additional $\gamma$ genes ($\gamma_{2.5}$); the auxiliary subunit
genes are discussed in more detail later (see section 1.3.4). Briefly, the LVA $\alpha_{I}$ subunit
genes were discovered as follows: homologous VDCC sequences were identified in
GenBank expressed sequence tag (EST) databases, providing numerous possible
sequences, further narrowed down according to known VDCC functional motifs (such as
the S4 and P-regions). Promising ESTs were sequenced and used as cDNA probes to
screen brain and heart cDNA libraries, resulting in the identification and cloning of the
$\alpha_{IG}$, $\alpha_{IH}$ and $\alpha_{II}$ clones. Expression of these clones has been performed in Xenopus oocytes
and HEK 293 cells (Cribbs et al., 1998; Perez-Reyes et al., 1998; Klugbauer et al., 1999a;
Lee et al., 1999a; Williams et al., 1999). The currents observed in these expression
systems were LVA, displayed slow deactivation (the closing process of the channel after completion depolarising step) and the single channel conductance was small (~8 pS). These characteristics clearly defined the $\alpha_{1G}$ VDCC subunits as the determinants responsible for the T-type VDCC currents (Kostyuk, 1999). However, studies by Meir and Dolphin (1998) have suggested that small conductance single channels observed in native neurons may also have been formed from other $\alpha_i$ subunits. In this study, Meir and Dolphin showed that HVA $\alpha_{1b}, \alpha_{1c}$ or $\alpha_{1e}$ VDCC subunit expression in COS-7 cells, particularly without auxiliary VDCC subunits, resulted in single channel currents in which both a low conductance (similar to T-type currents) and the expected high conductance single channel events were recorded. These results suggest that in addition to the obvious source of T-type currents arising from the $\alpha_{1G}$ clones, HVA $\alpha_i$ subunits may also produce a T-type like current particularly at small depolarisations.

A revised $\alpha_i$ VDCC nomenclature

The increasing numbers of $\alpha_i$ and auxiliary VDCC subunits has started to make the existing nomenclature complicated. The existing naming system using letters ($\alpha_i$ A-I, and S) is limited and does not provide an obvious or logical classification for the growing $\alpha_i$ VDCC subunit family. With these arguments in mind a group scientists working in the VDCC field have suggested a more logical naming and classification system (Ertel et al., 2000). This scheme is devised from that developed for the $K^+$ channel nomenclature (e.g. $K_{V1.1}$, $K_{V2.1}$, etc.). Using amino acid sequence homologies between VDCC $\alpha_i$ subunits a classification based on the phylogenetic tree that results from such sequence homologies has been suggested. A phylogenetic or family tree reflecting the sequence homologies has been copied from (Ertel et al., 2000) and is shown in Figure 1.2. According to this sequence homology-based system the previous divisions of HVA L-type, HVA non L-type and LVA channels will be maintained with each of these VDCC families being renamed as $Ca_{V1}$, $Ca_{V2}$ and $Ca_{V3}$. Family members within each of these
VDCC families will then be further defined by the order of cloning of each of the members. Thus, the HVA L-type family will be renamed as Cav1, with \( \alpha_{1S} \) being the first to be cloned within this family and so is renamed Cav1.1; consequently \( \alpha_{1C} \) is designated Cav1.2, \( \alpha_{1D} \) is Cav1.3 and \( \alpha_{1F} \) is Cav1.4. Splice variants for each \( \alpha_1 \) subunit can be further designated by lower case letters. The existing numerical nomenclature used for auxiliary VDCC subunits will remain unchanged. For example, the channel complex forming the neuroendocrine L-type current in HEK 293 cells studied here (see Chapter 4) at present is named \( \alpha_{1D}\beta_2/\alpha_{2B}\delta-1 \); under the new proposed naming system it would be renamed Cav1.3/\( \beta_3/\alpha_{2B}\delta-1 \). The new system provides a more logical framework for VDCC naming, allowing greater contingency for future cloning, with the name integrally providing more information about the clone (i.e. Ca\(^{2+}\) selectivity, voltage-dependence, phylogenetic class and cloning history).

Figure 1.2 Phylogenetic tree of \( \alpha_1 \) VDCC subunits with suggested nomenclature

Using the protein sequences for the more conserved transmembrane and pore region segments, the \( \alpha_1 \) VDCC subunits were arranged into a family tree according to homology of their protein sequences (using CLUSTAL analysis software). The new suggested VDCC family nomenclature is based on the protein sequence homology and corresponds well with the previously derived pharmacological and biophysical divisions. Thus the Cav1 family corresponds to the L-type VDCC, the Cav2 family corresponds to non L-type VDCC and Cav3 family to the low-voltage activated (LVA) VDCC. This figure was copied and annotated from Ertel et al., 2000.
The research that has contributed to the characterisation of the $\alpha_i$ Ca$^{2+}$ pore and selectivity filter provides strong evidence that the $\alpha_i$ channel pore creates a coordination site (or sites) for two or more Ca$^{2+}$ ions (see the review by Varadi et al., 1999). It has also been suggested that this may provide a mechanism whereby Ca$^{2+}$ ions coordinate in single file within the pore, using the electrostatic repulsion between neighbouring ions to create rapid influx of the ions; a theory that was first proposed for K$^+$ channels by Hodgkin and Keynes (1955).

Mutation studies on the $\alpha_i$ VDCC subunit revealed that the polypeptide linker between transmembrane repeats S5 and S6 on each domain (P-loops; see sites labelled ‘P’ in Figure 1.3) were important to the pore formation. These P-loops (alternatively described as SS1-SS2 sequences) were shown to contain glutamate residues in each of the four $\alpha_i$ subunit domains of HVA VDCC which were essential to the Ca$^{2+}$ conductance and selectivity (Tang et al., 1993; Yatani et al., 1994; Ellinor et al., 1995). Interestingly LVA channels have two aspartate residues in place of the glutamate residues found in HVA channels on domains III and IV (Cribbs et al., 1998; Perez-Reyes et al., 1998; Klugbauer et al., 1999a). It has been suggested that these critical residue changes in the LVA channel selectivity filter may account for the similarity in selectivity for Ba$^{3+}$ and Ca$^{2+}$ and for the small conductance observed in LVA VDCC currents. It is thought that the P-loops of each of the domains of the $\alpha_i$ VDCC subunit lie in a ring at the narrow point of the pore structure. The carbonyl groups on the glutamate residues in each P-loop are suggested to create a coordinated single (Armstrong & Neyton, 1991) or double (Almers & McCleskey, 1984; Hess & Tsien, 1984) Ca$^{2+}$ binding site(s), and hence selectivity for Ca$^{2+}$.

Similar to the pore regions found in VDCC are the pore regions that have been described in voltage dependent K$^+$ and Na$^+$ channels. The crystal structure of the bacterial (Streptomyces lividans) channel KcsA has been determined by Doyle et al. (1998), and it
has been suggested that the cone-like structure of the KcsA channel pore maybe applicable to the VDCC pore. In this analogous KcsA channel structure the base of the cone lies exofacially providing an aqueous pocket to incoming ions in which the selectivity filter lies. Following the region of the selectivity filter region the pore continues down to the pinnacle of the cone within the membrane, and then widens out once more into the intracellular space.

Channel activation and the voltage sensor

The channel activation, or opening of the channel, has its own distinct kinetics, a measurement of which provides another channel biophysical characteristic. By fitting an exponential (or multiple exponentials where different activation rates exist) to the activating phase of the VDCC current, a physical measure of the rate of activation can be obtained ($\tau_{\text{activation}}$). [However, in this study an alternative measure of activation, the time to 90% of peak current ($\text{tp}_{90\%}$) was used: this simpler measurement of activation overcomes any problems associated with fitting accuracy, with less assumptions required for mathematical fitting].

Studies performed in K$^+$ (Papazian et al., 1991) and Na$^+$ channels (Stuhmer et al., 1989) showed that mutations to the positively charged residues in the S4 transmembrane sequences held the key to the voltage-dependent activation. By analogy, mutational studies in VDCC $\alpha_1$ subunits concentrated on these areas. The regions of the VDCC $\alpha_1$ subunit that were shown to be essential for voltage-dependent activation are shown in the VDCC schematic represented in Figure 1.3. The primary sequence of the $\alpha_{1S}$ subunit (Tanabe et al., 1987) revealed the positive amino acid residues that constituted the generic voltage sensor of VDCC. These positive residues were found in regular arrays, at every third or fourth position, on the fourth transmembrane $\alpha$-helix (S4) in each of the four domains. Further evidence for these voltage-sensing residues was provided by the depolarisation of membranes containing voltage-dependent sodium channels (an analogous model for VDCC) which was hypothesised to cause movement of these
charged voltage-sensing residues and detected by recording tiny outward currents (Hodgkin and Huxley, 1952d), termed gating currents (Armstrong, 1981).

By the creation of αIS and αIC chimaeras, Tanabe et al. (1991) determined that domain I affected the speed of current activation. Specific arginine and lysine residues within the S4 sequences of domains I and III also affected the speed and voltage-dependence of current activation (Garcia et al., 1997). Additional sites within the αI VDCC subunit that were shown to be important to current activation were the IS3-IS4 (Nakai et al., 1994) and the IIIS5-IIIS6 linker (Dirksen et al., 1997).

**Channel inactivation**

Following complete channel activation, that is following the point at which peak channel current amplitude was observed, all VDCC currents (and most voltage dependent ion channels) display a progressive reduction of the current amplitude over the duration of the depolarising voltage potential. This characteristic is termed inactivation, and is a separate channel state in which channels can exist, along with the open (activated) and closed (deactivated) states. Like the activated state, the kinetics of inactivation can be mathematically described by fitting an exponential, or exponentials, to this inactivating phase of the current trace (for an example of such a fit to an inactivating phase of a current trace, see Figure 4.6A), which provides a measure of the inactivation rate (often referred to as the \( \tau_{\text{inactivation}} \)).

The process of inactivation provides a negative feedback inhibition mechanism by which the VDCC can reduce the influx of \( \text{Ca}^{2+} \) through the open channel. This self-regulating \( \text{Ca}^{2+} \) homeostasis mechanism has been shown to occur via two mechanisms: voltage-dependent and \( \text{Ca}^{2+} \)-dependent inactivation. Voltage-dependent inactivation was characterised by being unaffected by the charge-carrying ion (*i.e.* no change when using \( \text{Ca}^{2+} \) or \( \text{Ba}^{2+} \); Fox, 1981), and exhibits an acceleration of inactivation rates in response to increasing depolarising voltage steps. In contrast, \( \text{Ca}^{2+} \)-dependent inactivation is characterised by: a direct correlation with the entry of \( \text{Ca}^{2+} \) (Brehm & Eckert, 1978;
Eckert & Chad, 1984), which gives rise to the typical U-shaped dependence of inactivation rate on voltage, or release of Ca\(^{2+}\) (e.g. by caged Ca\(^{2+}\) release, Morad et al., 1988; or by IP\(_3\) release of stored Ca\(^{2+}\), Kramer et al., 1991). In addition, Ca\(^{2+}\)-dependent inactivation is affected by the charge-carrying ion (Ca\(^{2+}\) \(\gg\) Ba\(^{2+}\)), and is reduced by [Ca\(^{2+}\)]\(_j\) buffering agents (such as EGTA or BAPTA; Gutnick et al., 1989).

Heterologous expression of certain HVA channels (e.g. \(\alpha_{1A}\) and \(\alpha_{1E}\)) provide currents that exhibit mainly the voltage-dependent form of inactivation (though Ca\(^{2+}\)-dependent inactivation has been observed in expressed \(\alpha_{1A}\) currents, Lee et al., 1999; see discussion on calmodulin later, section 1.4.5). In contrast, expression of the \(\alpha_{1C}\) L-type calcium channel provides currents that are strongly inactivated by the Ca\(^{2+}\)-dependent form (though voltage-dependent inactivation is present to a lesser extent in these VDCC current forms, e.g. Bernatchez et al., 1998). Utilising these general trends for the main \(\alpha_1\) isoforms displaying each of the forms of inactivation, numerous studies using chimaeras of \(\alpha_{1A}/\alpha_{1E}\) (mainly voltage-dependent inactivation) with \(\alpha_{1C}\) (mainly Ca\(^{2+}\)-dependent inactivation), or by using site directed mutagenesis on these VDCC isoforms were performed. From these channel manipulations, several specific regions in the \(\alpha_1\) VDCC subunit have been shown to be important to the voltage-dependent or Ca\(^{2+}\)-dependent inactivation processes. These regions which create inactivation intrinsic to the \(\alpha_1\) subunit are highlighted in Figure 1.3, along with lists of the pertinent studies. However, not all VDCC isoforms conform to a specific type of inactivation: the N-type VDCC current display traits associated with both Ca\(^{2+}\)-dependent (Kasai and Aosakai, 1988) and voltage-dependent inactivation (Jones & Marks, 1989; Patil et al., 1998), creating confusion over the inactivation mechanism of this VDCC isoform. The voltage-dependent inactivation argument was strengthened recently by gating current studies performed on expressed N-type channels (Jones et al., 1999). This study also provided corroborative evidence for data suggesting that the levels of VDCC current inactivation were significantly greater in physiologically comparable multiple spike depolarisations,
Figure 1.3 α₁ VDCC sites important to the channel pore, activation and inactivation

Hydrophobicity plots of the α₁ subunit sequence predict a four domain structure (labelled I-IV, above the channel schematic). Each domain has six transmembrane helices (labelled S1-6 in domain I). An additional transmembrane loop lies between S5 and S6 on each domain, called the P-region (also known as the SS1-SS2 sequences) and form the pore and ion selectivity filter of the channel (labelled by P). The S4 helices in each domain contain residues forming the voltage sensor, which are denoted by +. Regions of the α₁ subunit critical to channel activation are white, whilst important sequences conferring inactivation traits upon the channel, are grey (voltage-dependent) and hatched (Ca²⁺-dependent). Studies that provided data suggesting sequences that were important in Ca²⁺-dependent and voltage-dependent are followed by (& V.).

- **Activation**
  - Tanabe et al., 1991
  - Nakai et al., 1994
  - Garcia et al., 1997

- **Pore**
  - Tanabe et al., 1987
  - Tang et al., 1993
  - Yatani et al., 1994
  - Dirksen et al., 1997

- **Inactivation - V. dependent**
  - Zhang et al., 1994
  - Yatani et al., 1994
  - Herlitze et al., 1997
  - Spaetgens and Zamponi, 1999
  - Stephens et al., 2000

- **Inactivation - Ca²⁺ dependent**
  - De Lion et al., 1995
  - Soldatov et al., 1997 (& V.)
  - Zhou et al., 1997
  - Cens et al., 1999 (& V.)

Mimicking action-potential pulse trains, when compared to the inactivation observed in standard square pulse depolarisations used in most studies (Patil et al., 1998). The authors suggest that an inactivation state is achieved with greater ease by channels from intermediate closed states, and thus trains of action-potential like depolarisations create increased opportunities to attain these intermediate closed states. This observation has led Patil et al. (1998) to the coining of the term ‘preferential closed-state inactivation’, which may be significant in the physiological implications for current inactivation.
The molecular mechanisms by which these processes of inactivation may arise are still unclear. Initial work on expressed VDCC currents (Zhang et al., 1994) argued against the blocking particle theory or ‘ball and chain’ based inactivation that had been previously observed in some K⁺ (Hoshi et al., 1990) and Na⁺ channels (Stuhmer et al., 1989). However, recent work has suggested that the polypeptide linker between domains I and II of the α₁ subunit, the I-II loop, may act as a ‘ball and chain’ blocking particle in both voltage-dependent and Ca²⁺-dependent inactivation of VDCC currents (Cens et al., 1999). The possibility of the I-II linker acting like a blocking particle is intriguing: it provides an explanation for the inactivation intrinsic to the α₁ VDCC subunit and also for the modulation of inactivation observed with the co-expression of auxiliary β VDCC subunits (see review by Castellano and Perez-Reyes, 1994; see also, Stephens et al., 2000, and section 1.3.4 below). It is suggested that since VDCC β subunits bind to the I-II loop, their presence are postulated to alter the 'mobility' of the I-II loop (Cens et al., 1999). Further studies have implicated the calcium binding protein calmodulin as both the sensor and mediator of the Ca²⁺-dependent form of inactivation (see review by Levitan, 1999); this will be discussed further in section 1.4.5.

1.3.4 VDCC auxiliary subunits

A summary of the auxiliary subunit isoforms, splice variants, and chromosomal and tissue localisation is provided in Table 1.3.
<table>
<thead>
<tr>
<th>Auxiliary subunit</th>
<th>Gene name (HUGO)</th>
<th>Chromosomal localisation (human)</th>
<th>Splice variants</th>
<th>Tissue localisation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>β₁</td>
<td>CACNB1</td>
<td>17q21-q22</td>
<td>β₁a</td>
<td>skeletal muscle, heart</td>
<td>Ruth et al. (1989); Pragnell et al. (1991); Powers et al. (1992); Williams et al. (1992b); Collin et al. (1993); Gregg et al. (1993); Illes et al. (1993)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β₁b</td>
<td>brain, heart</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β₁c</td>
<td>brain, heart, spleen</td>
<td></td>
</tr>
<tr>
<td>β₂</td>
<td>CACNB2</td>
<td>10p12</td>
<td>β₂a</td>
<td>heart, brain, lung</td>
<td>Hullin et al. (1992); Perez-Reyes et al. (1992); Williams et al. (1992b); Taviaux et al. (1997); Reimer et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β₂b</td>
<td>brain, heart</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β₂c</td>
<td>brain</td>
<td></td>
</tr>
<tr>
<td>β₃</td>
<td>CACNB3</td>
<td>12q13</td>
<td>β₃a</td>
<td>brain, smooth muscle, lung, ovary</td>
<td>Hullin et al. (1992); Castellano et al. (1993a); Collin et al. (1994); Yamada et al. (1995); Park et al. (1997); Reimer et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>β₃b</td>
<td>kidney</td>
<td></td>
</tr>
<tr>
<td>β₄</td>
<td>CACNB4</td>
<td>2q22-q33</td>
<td></td>
<td>brain, kidney</td>
<td>Castellano et al. (1993b); Taviaux et al. (1997); Escayg et al. (1998)</td>
</tr>
<tr>
<td>α₂δ-1</td>
<td>CACNA2A</td>
<td>7q21-q22</td>
<td>α₂δ-1₁</td>
<td>skeletal muscle</td>
<td>Ellis et al. (1988); Williams et al. (1992); Brust et al. (1993); Powers et al. (1994); Angelotti and Hofmann (1996)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α₂δ-1₂</td>
<td>brain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α₂δ-1₃</td>
<td>aorta</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α₂δ-1₄</td>
<td>aorta</td>
<td></td>
</tr>
<tr>
<td>α₂δ-2</td>
<td>CACNA2B</td>
<td>unknown</td>
<td>α₂δ-2₁</td>
<td>heart, pancreas, skeletal muscle, brain</td>
<td>Klugbauer et al. (1999a); Hobom et al. (2000)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α₂δ-2₂</td>
<td>brain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α₂δ-2₃</td>
<td>brain</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>α₂δ-2₄</td>
<td>brain</td>
<td></td>
</tr>
<tr>
<td>α₂δ-3</td>
<td>CACNA2C</td>
<td>unknown</td>
<td></td>
<td>brain</td>
<td>Klugbauer et al. (1999a)</td>
</tr>
<tr>
<td>γ₁</td>
<td>CACNG1</td>
<td>17q24</td>
<td></td>
<td>skeletal muscle</td>
<td>Bosse et al. (1990); Jay et al. (1990); Powers et al. (1993)</td>
</tr>
<tr>
<td>γ₂?</td>
<td>CACNG2</td>
<td>22q12-q13</td>
<td></td>
<td>brain</td>
<td>Letts et al. (1998); Black and Lennon (1999); Klugbauer et al. (2000)</td>
</tr>
<tr>
<td>γ₃?</td>
<td>CACNG3</td>
<td>16p12</td>
<td></td>
<td>brain</td>
<td>Black and Lennon (1999); Burgess et al. (1999); Klugbauer et al. (2000)</td>
</tr>
<tr>
<td>γ₄?</td>
<td>CACNG4</td>
<td>17q24</td>
<td></td>
<td>brain</td>
<td>Burgess et al. (1999); Klugbauer et al. (2000)</td>
</tr>
<tr>
<td>γ₅?</td>
<td>CACNG5</td>
<td>17q24</td>
<td></td>
<td>liver, kidney, heart, lung, skeletal muscle</td>
<td>Burgess et al. (1999); Klugbauer et al. (2000)</td>
</tr>
</tbody>
</table>
Following the purification of the skeletal muscle L-type calcium channel complex, the first β subunit to be cloned was the skeletal muscle associated β₁ (Ruth et al., 1989). Subsequently, a further three β subunits have been cloned: β₂ (Hullin et al., 1992; Perez-Reyes et al., 1992); β₃ (Hullin et al., 1992); and, β₄ (Castellano et al., 1993b). In addition to different β subunit clones, further variation is afforded the β subunit classes by splice variants (see Table 1.3; see also reviews by Castellano and Perez-Reyes, 1994; Hofmann et al., 1994; and, Birnbaumer et al., 1998). The primary sequence of the β subunits suggests a hydrophilic protein without glycosylation sites and lacking any predicted transmembrane sequences, and they have accordingly been shown to be localised on the intracellular surface of the cell membrane (Takahashi et al., 1987).

At present the exact secondary structure of the β subunits is not fully clarified, though recent modelling of the β₁b subunit has suggested the primary sequence could be divided into five putative domains (A-E), each formed of a mixture of α-helices and β-sheets (Hanlon et al., 1999). These five domains also approximately correlate with domains previously defined according to β subunit isoform alignments. The alignment of β subunit isoforms showed that between β subunit isoforms, domains 1,3 and 5 (A, C and E in Hanlon et al., 1999) showed least sequence conservation, whilst domains 2 and 4 (B and D in Hanlon et al., 1999) showed the greatest conservation (see reviews by Birnbaumer et al., 1998; and Walker and De Waard, 1998). Homology searches for the defined β₁ domains with other known structural motifs suggested that domain A was a PDZ (post synaptic disc-large zo-1)-like domain, domain B was a putative SH3 (src homology 3) domain and domain D was a guanylate kinase-like domain (Hanlon et al., 1999). Since domains B and D are significantly conserved between β subunits, it is possible that the SH3 and guanylate kinase-like domains observed in β₁ maybe functional motifs also found in the other β subunit isoforms. Another sequence that is conserved
throughout each of the β subunits has been termed the β-interaction domain (or BID). The BID sequence is 30 amino acids in length and lies at the N-terminal (proximal end) of the β subunit domain D (also referred to as domain 4, or the second conserved domain). The BID sequence allows the β subunit to interact and bind to the α1 VDCC subunit (De Waard et al., 1995). The corresponding binding site on the α1 subunit is termed the α-interaction domain (AID), with a minimum binding sequence of 18 amino acids, with 9 amino acids being conserved throughout all of the HVA VDCCs (QQXEXXLXGYXXW1XXXE, where X represent residues that are not totally conserved; see Walker and De Waard, 1998). The AID lies on a cytoplasmic loop linking domains I and II (the I-II loop) of the α1 subunit (see Figure 1.4, later). The interaction between the α1 and β subunits arises through these interaction domains, with mutations to either the AID or BID resulting in altered β subunit modulation of the VDCC current (De Waard et al., 1994; De Waard et al., 1996; Qin et al., 1996). In addition to the high affinity interaction site between the AID and the BID, a number of low affinity β interaction sites have also been discovered. These sites are specific to α1 and β subunit isoforms and include an interaction between the β4 subunit and the carboxyl or amino termini of α1A (Walker et al., 1998 and 1999), and a β subunit interaction with the carboxyl termini of the α1E (Tareilus et al., 1997; Qin et al., 1997).

The functional consequences of the β subunit interaction with the α1 subunits are numerous (see reviews by Birnbaumer et al., 1998; Walker and De Waard, 1998). The primary effects of β subunit co-expression with the α1 subunit are to:

i. Increase the peak amplitude of the VDCC current. This was attributed to an increase in the number of functional channels at the cell membrane (Lacerda et al., 1991; Perez-Reyes et al., 1992). Gating current measurements (Josephson & Varadi, 1996; Kamp et al., 1996) and immunocytochemical studies (Chien et al., 1995; Brice et al., 1997; Yamaguchi et al., 1998; Gao et al., 1999) have suggested a chaperoning-role for the β subunit in the translocation of α1 subunits.
to the cell membrane. Further evidence for the β subunit behaving as a chaperone was recently published (Bichet et al., 2000), in which the β subunit was shown to mask an ER retention sequence found on the α₁ subunit, and hence increase surface membrane translocation of the α₁ subunit. In addition to the chaperoning effect, work has shown that coupling between the α₁ voltage sensor and the pore opening was aided with β subunits present (Neely et al., 1993; Kamp et al., 1996).

ii. Produce a hyperpolarising shift in the activation of VDCC currents (Tomlinson et al., 1993; De Waard & Campbell, 1995; Stephens et al., 2000).

iii. Modulate both the current activation and inactivation (Lacerda et al., 1991; Castellano et al., 1993; Stea et al., 1993). Generally expression of β subunits results in an acceleration of these kinetic characteristics in co-expressed α₁ subunits. However, β₂α co-expression slows both current activation and inactivation, and the unusual properties observed with β₂α co-expression have been shown to be due to cysteine residues that are palmitoylated in the β₂α N-terminus (Chien et al., 1996). Mutation of these cysteine residues prevents palmitoylation, and results in a β₂α subunit with a reduced capability to slow current inactivation (Stephens et al., 2000).

iv. Interact with binding of the Gₓᵧ binding to the VDCC α₁ subunit, and hence affect the G-protein modulation of Gₓᵧ subunits on VDCC currents. Studies have suggested that β subunit co-expression reduces the G-protein Gₓᵧ inhibition of VDCC currents (Roche et al., 1995; Bourinet et al., 1996); corollary experiments, where levels of VDCC β subunits were reduced by a β subunit anti-sense oligonucleotide, showed an increased inhibition by Gₓᵧ modulation (Campbell et al., 1995b). Thus, these studies argue that the presence of VDCC β subunits reduces Gₓᵧ inhibition of calcium channel currents. However, recent evidence
within Prof. Dolphin's lab has argued that although varying the amount of VDCC \( \beta \) subunits produces effects on the voltage-dependence of G-protein modulation (Canti et al., 2000), in the complete absence of \( \beta \) subunits voltage-dependent G-protein modulation of \( \alpha_{1\text{In}} \) is lost. This appears to be due to the \( \beta \) subunit facilitating the removal of \( G_{\beta} \) from the \( \alpha_1 \) subunit at depolarised potentials (Meir et al., 2000; and see Chapter 5).

\( \alpha_2\delta \) subunits

Once again, the early protein purification of the L-type skeletal muscle provided the first sequence of the \( \alpha_2\delta \) subunit (Ellis et al., 1988; this isoform is now called \( \alpha_2\delta-1 \)). The \( \alpha_2\delta-1 \) protein is heavily glycosylated, and is formed by proteolytic cleavage of a single gene product, the two halves linked by disulphide bridges. The primary sequence suggests that the \( \delta \) subunit forms a transmembrane domain which anchors or tethers the \( \alpha_2 \) subunit. The \( \alpha_2 \) subunit does not appear to have any transmembrane sequences, and lies entirely extracellularly (see subunit arrangement in Figure 1.1) (Wiser et al., 1996a).

To date the single \( \alpha_2\delta-1 \) gene has been shown to produce five mRNA species due to splice variations (\( \alpha_2\delta-1\text{a-e} \); Brust et al., 1993), and they appear to display tissue specific distributions (see Table 1.3; Angelotti and Hofmann, 1996). Three splice variations have been shown to occur in the \( \alpha_2\delta-2 \) gene (\( \alpha_2\delta-2\text{a-c} \); Hobom et al., 2000).

Functional effects of co-expressing the \( \alpha_2\delta-1 \) with \( \alpha_1 \) subunits includes an increase in peak current amplitude (Williams et al., 1992a; Tomlinson et al., 1993; Shistik et al., 1995) and an acceleration of the rate of activation (Singer et al., 1991; Tomlinson et al., 1993). However, the most obvious and greatest range of effects are observed when additionally co-expressed with \( \beta \) subunits, often acting synergistically with the functional effects that the \( \beta \) subunit provides (see review by Walker and De Waard, 1998). The mechanistic explanation for the \( \alpha_2\delta-1 \) effects is still unclear though some studies have provided evidence for \( \alpha_2\delta-1 \) functional mechanisms. The increased
current density observed with $\alpha_2$δ-1 co-expression has been shown in the *Xenopus* oocyte expression system to arise due to greater targeting of co-transfected $\alpha_1$ subunits to the cell membrane (Shistik *et al.*, 1995). When the $\alpha_2$δ-1 subunit was co-expressed with $\alpha_{1C}$ in HEK 293 cells, the gating charge movement increased during channel activation, which was subsequently observed to increase the conductance in the $\alpha_{1C}/\alpha_2$δ-1 channels (Bangalore *et al.*, 1996). This may suggest a role for the $\alpha_2$δ-1 subunit in which the coupling of the voltage-sensor with the channel activation is more efficient or more sensitive to depolarisation. However, similar gating charge effects were also observed with $\alpha_{1E}$ co-expression with $\alpha_2$δ-1, though the increased conductance that was apparent with $\alpha_{1C}$ was not observed (Qin *et al.*, 1998).

The therapeutic drug gabapentin acts as an anticonvulsant and has been shown to have a high affinity binding site on the $\delta$ subunit of $\alpha_2$δ-1 (Brown & Gee, 1998). However, the mechanism of the anticonvulsant behaviour of gabapentin in relation to $\alpha_2$δ-1 association with HVA currents is still unclear: one study has shown an inhibitory effect upon HVA currents in response to gabapentin application (Stefani *et al.*, 1998), whilst another study showed no effect (Taylor *et al.*, 1998).

Recently the $\alpha_2$δ family was expanded by two further members using a sequence database search method (Klugbauer *et al.*, 1999b). The $\alpha_2$δ-2 and $\alpha_2$δ-3 subunits only display 30% and 56% primary sequence homology to the $\alpha_2$δ-1 isoform. However, the secondary structure of each, particularly hydrophobicity plots and numbers and positions of N-glycosylation sites, suggest a stronger homology to $\alpha_2$δ-1 than provided by the primary structure. In addition the initial functional data of $\alpha_2$δ-3 co-expression with $\alpha_{1C}/\beta_{2a}$ VDCC subunits displays close similarity to that observed with $\alpha_2$δ-1 co-expression (Klugbauer *et al.*, 1999b). Functional effects on the current kinetics and biophysics of the $\alpha_2$δ-2 were also recently been observed when co-expressed with $\alpha_{1A}$, $\alpha_{1C}$, $\alpha_{1E}$, and $\alpha_{1G}$ VDCC subunits (Platzer *et al.*, 2000).
**γ subunits**

The biochemical characterisation of the L-type calcium channel skeletal muscle showed the presence of a γ subunit (Jay et al., 1990). Until recently, functional channels for other VDCC types were considered to be composed of α₁, α₂δ and β subunit complexes (though functional channels can be formed of the α₁ subunit alone, e.g. see Meir and Dolphin, 1998). The functional expression of γ subunit with α₁C has been shown to produce small effects upon the channel biophysics, such as: increased peak amplitude; a hyperpolarising shift in the voltage-dependence of activation; and accelerated activation kinetics, though these effects were only observed when β subunits were additionally co-expressed (Wei et al., 1991).

The pervading opinion that the skeletal muscle L-type VDCC was the only VDCC complex that included the γ subunit has recently been challenged: four new putative γ subunits were discovered, called Y2.5 (Letts et al., 1998; Black & Lennon, 1999; Burgess et al., 1999; Klugbauer et al., 2000). Unlike the original γ subunit (renamed γ₁) which was almost exclusively found in skeletal muscle (Jay et al., 1990), three of the putative subunits (γ₂₋₄) were found in neuronal tissue and γ₅ was found in several non-neuronal tissues (see Table 1.3)(Klugbauer et al., 2000). The γ₂ subunit appears to have a neurological function since the γ₂ subunit was discovered due to a genetic mutation in neurological mutant mouse lines called stargazer (Letts et al., 1998) and waggler (Chen et al., 1999). The primary sequence homology between murine γ₁ and the four new putative γ subunits is less than 25% in each case (Klugbauer et al., 2000); the human orthologues of γ₂ and γ₅ (Black and Lennon, 1999) display even lower sequence homologies when compared with γ₁ (18% and 17% respectively). However, hydrophobicity plots and N-glycosylation sites for γ₂.5 are very similar to γ₁, providing evidence that these additional suggested γ family members are in fact γ subunits, despite the low primary sequence homologies (Black & Lennon, 1999; Burgess et al., 1999).
possible effects that these putative new γ family members have upon the VDCC complex, and hence currents, still requires extensive characterisation, though functional data of γ co-expression with α₁βδ/α₂δ complexes does suggest that γ subunits may have a modulatory role. Of the new γ subunits co-expressed with α₁A and α₁C channels, the γ₂ subunit (and to a lesser extent γ₄) has been shown to produce modest, though significant, effects upon channel biophysical characteristics such as steady-state inactivation and rates of current activation and inactivation (Letts et al., 1998; Klugbauer et al., 2000). In addition the γ₂ and γ₄ subunits when co-expressed with the T-type α₁C subunit significantly retarded the rate of recovery from voltage-dependent inactivation; the γ₅ subunit accelerated both the rate of current activation and inactivation of α₁C currents (Klugbauer et al., 2000).

### 1.4 VDCC modulation

The operation of the VDCC current is a complex balance of intrinsic activation and inactivation of the α₁ subunit, combined with the manifold effects of differing types and combinations of the associated auxiliary VDCC subunits. The critical role of Ca²⁺ in the numerous activities of a variety of cell types (see section 1.2, for just a few examples) and the prime role that VDCCs play in the global cellular [Ca²⁺]ᵢ homeostasis, makes the control of the Ca²⁺ entry via VDCCs an imperative focal point of several mechanisms. Thus, in addition to the inherent controls provided by the α₁/auxiliary subunit complex, a number of cellular proteins also interact, modulate and shape the VDCC current. Figure 1.4 summarises the main modulatory proteins and their interaction sites on the α₁ subunit. Modulation of the current arises through: direct G-protein subunit interactions (both Gₚγ dimers and Gₐo have modulatory roles); phosphorylation of channel amino acid residues by protein kinase C (PKC) and cAMP-dependent protein kinase A (PKA); binding of SNARE (soluble NSF attachment protein receptors) proteins provides both channel modulation and binding events which allow spatial control of neurotransmitter
exocytosis; and, binding between the α₁ subunit and ryanodine receptors (RyR) provides an intimate relationship to control the Ca²⁺-induced Ca²⁺ release provided by these ER membrane proteins. It is these protein interactions and subsequent VDCC modulations that will be discussed in the following sections.
Figure 1.4 $\alpha_1$ VDCC sites important to the channel modulation by G-proteins, phosphorylation and other protein interactions

The four domain repeat structure (each consisting of S1-6 transmembrane domains; see Figure 1.3) of the generic $\alpha_1$ VDCC subunit has been annotated in this schematic to show the main points of interaction that have been determined by mutation and chimaeric studies on various HVA $\alpha_1$ isoforms. The regions are shaded and labelled as follows: $G_{\beta\gamma}$ interaction sites are in light grey boxes; sites important to protein kinase-C phosphorylation (PKC) on the $\alpha_1$ subunit are highlighted by a white star; the $\alpha$-interaction domain (AID), the point at which the VDCC $\beta$ subunit binds, is denoted by a hatched box; the binding/interaction site for the exocytotic SNARE proteins (e.g. syntaxin, SNAP 25, synaptotagmin) are highlighted by a lattice patterned box; sites interacting with the ryanodine receptor (Ry-R) are boxed and shaded in dark grey; the site of cAMP-dependent protein kinase-A (PKA) phosphorylation is highlighted by a white cross; and, the $G_{\alpha_\circ}$ interaction site is defined by a white box. The positions of each of these modulation sites are approximate, and designate sites determined on several different $\alpha_1$ subunits (see the text for the specific $\alpha_1$ subunit affected for each modulation site).
1.4.1 Heterotrimeric Guanine-nucleotide binding proteins (G-protein)

G-protein families and diversity

The G-protein is an heterotrimeric complex consisting of a $G_\alpha$, $G_\beta$ and $G_\gamma$ (though the $G_\beta$ and $G_\gamma$ are functionally effective as a $G_{\beta_\gamma}$ dimer). The $G_\alpha$ subunits are divided into four families: $G_{\alpha_1}$, $G_{\alpha_i/0}$, $G_{\alpha_q}$ and $G_{\alpha_{12}}$. A number of different isoforms, and splice variants of isoforms, of the $G_\alpha$ subunit have been shown to exist, resulting in at least 23 different $G_\alpha$ subunits encoded by at least 16 different genes (Simon et al., 1991; Nurnberg et al., 1995). The $G_\beta$ subunit is encoded by five different genes ($G_{\beta_1-5}$), and 11 $G_\gamma$ subunit genes have been cloned ($G_{\gamma_{1-12}}$, only 11 because $G_{\gamma_6}$ was later called $G_{\gamma_2}$). There are numerous permutations of these two G-protein subunits (though not all permutations appear to occur naturally) to provide a diverse population of $G_{\beta_\gamma}$ dimers (Yan et al., 1996; Clapham & Neer, 1997; Hildebrandt, 1997).

The G-protein activation-inactivation cycle

Activation of the trimeric G-protein causes GTP to preferentially bind to the $G_\alpha$ subunit, releasing pre-bound GDP, and creating two active G-protein species from the inactive trimer: $G_\alpha$-GTP and $G_{\beta_\gamma}$ dimers, both of which can modulate the VDCC $\alpha_1$ subunit (as well as a number of other transduction pathways; see Neer, 1995). The G-protein cycle is completed by an intrinsic $G_\alpha$ GTPase activity (Carty et al., 1990; Bourne et al., 1990), which allows the hydrolysis of the $G_\alpha$ bound GTP to GDP, recycling $G_\alpha$-GDP moieties and allowing re-binding of the active $G_{\beta_\gamma}$ dimer. Like the regulation observed in VDCC $\alpha_1$ subunits, the intrinsic GTPase activity which controls the G-protein activity is additionally controlled by external proteins. The rate of hydrolysis of the $G_\alpha$ subunit GTPase can be accelerated (Srinivasa et al., 1998; Jeong & Ikeda, 2000) by a family of proteins called regulators of G-protein signalling (RGS). The RGS proteins, therefore,
can reduce the time the G-protein is effectively active (desensitisation, see Diverse-Pierluissi et al., 1999). The VDCC β subunit, too, has been shown to exhibit an inhibitory role on G-protein activity, again using an acceleration of the GTPase activity associated with the Gα subunit to reduce the duration of the G-protein activation (Campbell et al., 1995a). This effect may be related to the VDCC β subunit mediated enhancement of the off-rate of Gp, at depolarised potentials (Canti et al., 2000; Meir et al., 2000).

**G-protein coupled receptors (GPCRs)**

The activation of the inactive G-protein trimer within cells is performed by a family of cell membrane proteins called G-protein coupled receptors (GPCRs; see reviews by Gudermann et al., 1997; Wess, 1997). GPCRs are seven transmembrane proteins (7TM) that transduce an external ligand binding event into the intracellular activation of trimeric G-proteins. The ligands that routinely activate GPCRs are neurotransmitters and hormones, though a number of pharmacological agents and toxins have been discovered to mimic naturally occurring ligand binding. There are estimated to be over a thousand GPCRs, though they have a limited number second messenger pathways (e.g. protein kinases, phospholipases, phosphatases) and ion channels which transduce the external signal into intracellular signalling (see review by Birnbaumer et al., 1990). Due to their critical role in transducing numerous neurotransmitter and hormonal signalling pathways, GPCRs have been the target of a multitude of therapeutic drugs. The importance of targeting GPCR activity by therapeutic compounds is highlighted by the estimation that up to a third of prescribed, clinical drugs act at GPCRs (see Stadel et al. 1997).

In this study three GPCRs were used to investigate the action of GPCR activated G-proteins on VDCC currents: the dopamine D2 receptor (D2); the muscarinic acetylcholine subtype 4 receptor (M4); and, the somatostatin subtype 2 receptor (sst2). In addition to activating a number of different second messenger pathways each of these GPCRs (D2, M4 and sst2) have been shown to inhibit calcium currents in several studies.
The D2 receptor is member of a family of five dopamine receptors (D1-5; for reviews see Sokoloff and Schwartz, 1995; Missale et al., 1998). According to pharmacology, activation of second messenger pathways and transmembrane sequence homologies the dopamine receptor family is subdivided into two main families: the D1-like family consisting of the D1 and D5 (alternatively labelled D1b) isoforms; and the D2-like family consisting of D2-4 isoforms. The D2 isoform has two functionally expressed splice variants: a short D2 (D2S) and a longer form (D2L) with an additional 29 amino acids inserted on the third intracellular loop connecting transmembrane domains V and VI (Giros et al., 1989; Monsma et al., 1989). Both forms have been used in this study to investigate the effects of GPCR activation on VDCC currents: a GH4C1 cell line stably expressing the human D2S receptor was studied (see Chapter 3); and a rat D2L receptor was investigated by transient transfection of HEK 293 (see Chapter 4) and COS-7 cells (see Chapter 5). Despite the third intracellular loop being important in the GPCR interaction with G-proteins only modest functional differences have been observed between the two D2 splice variants (see review by Missale et al., 1998). As well as the natural catecholamine agonist ligand dopamine, dopamine receptors have a number of pharmacological agents have been shown to be dopamine receptor agonists (e.g. quinpirole was used in this study) and antagonists (e.g. (-)-sulpiride, haloperidol).

The muscarinic acetylcholine (M) receptors are also a family consisting of five members (M1-5) (see review by Caulfield and Birdsall, 1998). In addition, recently a patent application for a putative sixth M receptor gene, m6, was made (Goodearl, 1999); however, as yet no functional data or pharmacology has been published. The M4 receptor used in this study was an endogenous receptor found in the GH4C1 cell line (see section 1.6). Amongst the G-protein effects of M4 activation by the natural ligand acetylcholine or, for example, the non-specific agonist carbachol used in this study, is the inhibition of
L-type VDCC, which has been shown to require activated $G_{a0}$, $G_{a2}$, and $G_{a3}$ for normal inhibition of the current (Ye et al., 1999).

Once again, the somatostatin receptors are a GPCR family with five clonal members (sst1-5) (see review by Hoyer et al., 1995). Somatostatin (SS or SST) is a natural ligand, and was also known as somatotropin-release-inhibiting factor (SRIF). There are two forms of SS: a 14 amino acid form (SS-14; used in this study) and a 28 amino acid form (SS-28), both formed by proteolytic cleavage of a preprohormone (Patel & Galanopoulou, 1995). Classically, the SS-14, -28 polypeptides were the only natural ligands: however, two new ligands were discovered cortistatin-14 and cortistatin-29, again the products of proteolytic cleavage of a preprohormone (De Lecea et al., 1996).

Several studies have shown the activation of sst2 receptors (endogenously expressed in GH4C1 and HEK 293 cells, and used in this study) results in inhibition of VDCC currents, and using G-protein antisense oligonucleotides the $G_{a2}$, $G_{p1}$, and $G_{y3}$ subunits were shown to be responsible for this modulatory response (Kleuss et al., 1991; Kleuss et al., 1992; Kleuss et al., 1993; Degtiar et al., 1996).

**Useful toxins and compounds used in the investigation of G-protein effects**

Two bacterial exotoxins, which act by ADP-ribosylation, have been routinely used in the dissection of G-protein pathways (see Birnbaumer et al., 1990). The toxin produced by *Vibrio cholerae*, cholera toxin or CTX, using GTP ADP-ribosylates an arginine residue on the $G_{as}$ subunit preventing the intrinsic GTPase activity of the $G_{as}$ subunit, locking the G-protein in the active form. In a similar ADP-ribosylation catalytic reaction, though requiring ATP as a co-substrate, pertussis toxin or PTX (from the bacteria *Bordetella pertussis*) ADP-ribosylates a cysteine residue specifically found on the C-termini on the $G_{ai/o}$ family of subunits, disrupting the G-protein/GPCR interaction. Thus, these two toxins CTX and PTX can allow differentiation between the $G_{as}$ and $G_{ai/o}$ pathways respectively.
Since GTP and GDP are key components in the G-protein activation-inactivation cycle (see above), using non-hydrolysable analogues of each can push the cycle to conditions favouring G-protein activation or inactivation (see Figure 1.5, later). Thus, guanosine-5'-0-thiotriphosphate (GTP-γS) maintains G-proteins in an activated state, whilst the converse occurs with guanosine-5'-0-(2-thiodiphosphate) (GDP-βS).

1.4.2 Membrane delimited G-protein modulation of VDCCs

On activation of a GPCR by an agonist the inactive heterotrimeric G-protein binds GTP and forms two active species: Ga-GTP and Gβγ. Both these active G-protein particles are capable of activating pathways in which intracellular second messengers are activated or formed (see sections 1.4.4 and 1.4.5, later). In addition to these second messenger pathways the Ga-GTP and Gβγ particles can also act directly upon the VDCC. Since the G-protein complex is closely associated with the membrane bound GPCR that activates it, and the VDCC is also a membrane protein, this direct pathway has been called membrane-delimited modulation, differentiating it from the cytosolic based second messenger pathways. During initial studies exemplifying the membrane-delimited pathway, compelling evidence was provided by cell attached recordings, in which G-protein modulation of calcium channel current was only observed when the agonist was in the patch pipette (Forscher et al., 1986). No effect was observed when the agonist was applied to the rest of the cell surface, and hence a clear indication of 'tight coupling' between GPCR and VDCC (see Brown, 1993). Direct binding of Gβγ and Ga to particular sites on the VDCC α1 subunit of certain HVA isoforms (see below) provided the convincing evidence to clarify the membrane-delimited hypothesis suggested by early electrophysiological recordings.
Voltage-dependent modulation ($G_{\beta\gamma}$ subunits)

The classic electrophysiological characteristics of membrane-delimited, voltage-dependent G-protein modulation are shown in Figure 1.5. The typical characteristics include (see reviews by Dolphin, 1995, 1998):

(i) current inhibition across a range of voltage depolarisations (though particularly prevalent at lower voltage depolarisations).

(ii) a slowed rate of the current activation (again, more apparent at lower voltages).

(iii) a depolarising shift in the IV relationship.

(iv) a temporary removal of the G-protein inhibition and associated biophysical properties by a large depolarising pre-pulse (> +100 mV), a process termed pre-pulse facilitation.

(v) the pathway is sensitive to PTX ribosylation, suggesting that the free $G_{\beta\gamma}$ are released specifically from $G_{\alpha\omega}$ subunits.

VDCC currents displaying this form of G-protein modulation were described as ‘reluctant’, whilst the temporary removal observed during pre-pulse facilitation gave rise to a ‘willing’ channel state (Bean, 1989).

Initial studies into voltage-dependent G-protein modulation suggested that the $G_{\alpha}$ subunits were responsible for the observed effect (Hescheler & Schultz, 1993). However, substantial evidence has since been unearthed showing that the typical characteristics listed above are observed in N- and P/Q type currents when $G_{\beta\gamma}$ subunits are over-expressed (Herlitze et al., 1996; Ikeda, 1996). These studies were supplemented by investigations showing direct binding of radio-labelled $G_{\beta\gamma}$ to residues in the intracellular loop connecting domain I and domain II (I-II loops) of $\alpha_{1A}$ and $\alpha_{1B}$ VDCC subunits (De Waard et al., 1997; Zamponi et al., 1997). It is now widely accepted that the $G_{\beta\gamma}$ subunits perform the voltage-dependent G-protein modulatory role, with binding of activated the $G_{\beta\gamma}$ subunit(s) to the $\alpha_{1}$ VDCC subunit causing the characteristics listed above.
Figure 1.5 Characteristics of Gβγ modulation of VDCC currents

A cartoon displaying the characteristics of Gβγ, modulation of VDCC currents. **Control** (far left): shows a cartoon current elicited by voltage (V) depolarisation during control conditions (CTRL; the cartoon channel and resulting current trace are shaded grey). Gβγ, **inhibition** (middle): activation of trimeric G-proteins by GPCR ligands (e.g. Quinpirole, Quin; somatostatin-14, SS-14) or by a GTP analogue (GTP-γS) increases the level of free Gβγ (black oval). Increased levels of free Gβγ causes a negative (-ve) modulatory effect upon the channel current (the Gβγ-modulated cartoon channel and current trace are shaded black). The negative modulatory effects of Gβγ include: current inhibition; a slowed current activation; and, a positive shift in the voltage-dependence of activation (positive shift of the IV relationship). The levels of free Gβγ can be reduced by maintaining the inactive trimeric G-protein using a GDP analogue (GDP-βS) or pertussis toxin (PTX). **Pre-pulse facilitation** (far right): the Gβγ bound to the modulated VDCC can be temporarily removed by a large depolarising pre-pulse (indicated by the larger lightning symbol). The removal of the Gβγ reverses the associated inhibitory effects, resulting in a current enlargement (pre-pulse facilitation) and an acceleration of the current activation. The temporarily facilitated cartoon channel (white) and the current following the pre-pulse (+PP, dotted trace) are shown; the Gβγ inhibited current without a preceding pre-pulse is shaded black and labelled noPP.
Concomitant with this hypothesis, the temporary removal of bound $G_{\beta y}$ by large pre­pulses results in the characteristic facilitation, and as might be predicted by the $G_{\beta y}$ binding hypothesis, the subsequent rate of re-block ($G_{\beta y}$ rebinding) is dependent on the concentration of free $G_{\beta y}$ subunits (Elmslie & Jones, 1994; Zamponi & Snutch, 1998; Stephens et al., 1998a). The $G_{\beta y}$ modulation is commonly observed in the non L-type HVA channels ($\alpha_{1A}$, $\alpha_{1B}$ and $\alpha_{1E}$). L-type currents (both native and heterologous expression of $\alpha_{1C}$ channels) are largely resistant to this form of G-protein modulation, though there is significant bank of evidence that shows that the neuroendocrine L-type current does exhibit the characteristics associated with this form G-protein modulation (Haws et al., 1993; Piros et al., 1996; Tallent et al., 1996; Degtiar et al., 1997). This G-protein modulation of neuroendocrine L-type currents performs a prominent physiological function controlling the rate of hormone and neurotransmitter release from these secretory cells. For instance tumour pituitary cell lines, which have been studied extensively as models for neurosecretory cells, have shown that GPCR activation by SS-14 results in VDCC current inhibition and a subsequent reduction of hormone exocytosis from these cells (Richardson, 1983; Luini et al., 1986).

A number of studies using $\alpha_1$ VDCC subunit mutants and chimaeras have provided evidence for three sites on the $\alpha_1$ subunit that are important to $G_{\beta y}$ modulation (see Figure 1.4). Within the I-II loop critical $G_{\beta y}$ binding residues have been defined: the consensus binding motif is -QXXER- and is present in all the non L-type HVA VDCC ($\alpha_{1A}, \alpha_{1B}$ and $\alpha_{1E}$) (De Waard et al., 1997; Page et al., 1997; Qin et al., 1997; Zamponi et al., 1997). These critical $G_{\beta y}$ binding residues and additional residues within the I-II loop overlap with the $\alpha$-interaction domain (AID), the point at which the VDCC $\beta$ subunit interacts with the $\alpha_1$ subunit (see Figure 1.4). Due to this overlap it has been suggested that VDCC $\beta$ subunit and the $G_{\beta y}$ dimer compete for binding to this site on the VDCC $\alpha_1$ subunit, explaining the apparent antagonistic effect of VDCC $\beta$ subunits on $G_{\beta y}$ modulation (Bourinet et al., 1996). However, this hypothesis of competition and
subsequent antagonism by the VDCC β subunit has been challenged recently (Meir et al., 2000; see also Chapter 5). Two further sites that have been shown to be important to the G\textsubscript{\beta\gamma} modulation of the non L-type HVA VDCC are the α\textsubscript{1} VDCC subunit C-terminus (Zhang et al., 1996; Qin et al., 1997) and the N-terminus (Page et al., 1998; Canti et al., 1999)(see Figure 1.4). These studies used chimaeras of each of the typically G\textsubscript{\beta\gamma} modulated channels (α\textsubscript{1A,B,E}) spliced with the non modulated α\textsubscript{1C} to define the specific areas that are necessary for the G-protein modulation of the channel. Page et al. (1998) showed that 11 amino acids in the N-terminus were important to G\textsubscript{\beta\gamma} modulation, three of which were critical. Qin et al. (1997) revealed a 38 amino acid residue stretch within the C-terminus to be similarly important in G\textsubscript{\beta\gamma} modulation. Figure 1.6 shows sequence alignments for the human α\textsubscript{1B}, α\textsubscript{1C} and α\textsubscript{1D} N-termini and I-II loops. (The C-termini alignments were performed, though were not shown because each of the α\textsubscript{1} subunits show poor conservation in this area, making comparisons ineffectual). Though there are some similarities between the G\textsubscript{\beta\gamma} modulated α\textsubscript{1B} and the non-modulated α\textsubscript{1C} sequences, in key areas (shaded in grey) there is sufficient heterogeneity to account for the difference in G\textsubscript{\beta\gamma} modulation. As might be predicted the α\textsubscript{1C} and α\textsubscript{1D} show strong conservation throughout the alignments, and particularly in the crucial grey-shaded areas responsible for G\textsubscript{\beta\gamma} modulation; this suggests that the α\textsubscript{1D} channel currents, like the α\textsubscript{1C} channels, may be predicted to be resistant to G\textsubscript{\beta\gamma} modulation (and was borne out by the data described later, see Chapter 4). In the G\textsubscript{\beta\gamma} modulated α\textsubscript{1} isoforms (α\textsubscript{1A,B,E}) the G\textsubscript{\beta\gamma} binding motif within the I-II loop is -QQIER- (fitting the consensus motif of -QXXER-), whilst the non-modulated α\textsubscript{1C} (and incidentally α\textsubscript{1D}) isoform has -QQLEE-. Studies in which point mutations were performed such that loss of G\textsubscript{\beta\gamma} modulation was shown by the mutation -QQIER\rightarrow E- in α\textsubscript{1A} and α\textsubscript{1B}; whilst gain of modulation was displayed when the corollary mutation was performed in the α\textsubscript{1C} (i.e. -QQLEE\rightarrow R-)(De Waard et al., 1997; Herlitze et al., 1997). However, the work by Zhang et al. (1996) disputes these claims, suggesting
Figure 1.6 Amino acid sequence alignments for the N-termini and I-II loops of the human $\alpha_{1B}$, $\alpha_{1C}$ and $\alpha_{1D}$ subunits

Single code amino acid alignments are shown for the human $\alpha_{1B}$ (Genbank accession no. m94172), $\alpha_{1C}$ (z34809) and $\alpha_{1D}$ (m76558; used in this study, see Chapter 4). Alignments are shown between the N-termini (upper alignment) and the I-II loop (lower alignment). In each alignment residues that have been shown to be important in the $G_{\beta\gamma}$ modulation of VDCC currents have been shaded in grey. Within these important grey highlighted areas, specific residues essential to $G_{\beta\gamma}$ modulation are designated by an asterisk (*) above the sequences and boxed. Two or more identical residues within the alignment are denoted by bold, capital letters; non-identical residues are in lower case letters. The sequence numbering (above alignments), for clarity, refers only to the start of the alignments, and not the absolute numbering of each individual sequence.

**HUMAN N-TERMINI ALIGNMENTS**

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**HUMAN I-II LOOP ALIGNMENTS**

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</table>

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that the I-II loop had no effect on \( G_{\beta_y} \) modulation. Clearly, strong evidence exists for each of three important \( G_{\beta_y} \) modulation sites elucidated, and it is likely that a combination of these sites is likely to be required to provide the full complement of \( G_{\beta_y} \) modulation characteristics.

The mechanism determining specificity of the signal transduction provided by the \( G_{\beta_y} \) modulatory pathway has yet to be clarified. The specificity does not appear to be due to specific combinations of \( G_{\alpha} \) subunits with \( G_{\beta_y} \) dimers, since the binding between subunits appears to be promiscuous (Jeong & Ikeda, 1999). Another mechanism of specificity has been proposed to arise from the particular combinations of \( G_{\beta} \) with \( G_{\gamma} \) subunits, and a study has been performed which shows that particular \( G_{\beta} \) subunits (\( G_{\beta_1}, G_{\beta_2} \) and small effects with \( G_{\beta_5} \)) allowed the observation of voltage-dependent, \( G_{\beta_y} \) modulation; other \( G_{\beta} \) subunits did not support this modulation (\( G_{\beta_3} \) and \( G_{\beta_4} \)) (Garcia et al., 1998). However, further work is required to confirm this hypothesis since more recently, a range of heterodimers formed with \( G_{\beta_1-5} \) and various \( G_{\gamma} \) subunits were all shown to be capable of displaying characteristic voltage-dependent, \( G_{\beta_y} \) modulation (Ruiz-Velasco & Ikeda, 2000).

**Modulation by \( G_{\alpha} \) subunits**

Although the voltage-dependent, membrane-delimited modulation of VDCC by G-proteins has been shown to be caused by \( G_{\beta_y} \), other forms of G-protein modulation have also been attributed to direct interaction of \( G_{\alpha} \) subunits with the VDCC \( \alpha_i \) subunit. A voltage-independent form of \( \alpha_{iA} \) and \( \alpha_{iB} \) channel current inhibition was observed in oocytes when co-expressed specifically with \( G_{\alpha_{01}} \) and \( G_{\alpha_{i3}} \) respectively (Furukawa et al., 1998a,b). This group also showed that a \( G_{\alpha_{06}} \) subunit binding site in the C-terminus (see Figure 1.4) of the \( \alpha_{iA} \) and \( \alpha_{iB} \) subunits was responsible for the observed modulation. Ikeda and colleagues have also shown that other \( G_{\alpha} \) subunits can modulate VDCC currents: over-expression of \( G_{\alpha_{2}} \) in sympathetic neurons was shown to inhibit N-type
channels by a voltage-dependent, PTX insensitive pathway (Jeong & Ikeda, 1998); and, activation of M1 receptors in sympathetic neurons revealed a PTX insensitive, voltage-independent pathway that required the presence of active $G_{\alpha}$ and $G_{\beta\gamma}$ (Kammermeier et al., 2000). Another voltage-dependent, PTX insensitive pathway has also been observed to inhibit N- and P/Q-type VDCC currents in chromaffin cells, probably by activating $G_{\alpha}$ via histamine $H_1$ receptors (Currie & Fox, 2000).

1.4.3 VDCC phosphorylation

There are several modulatory pathways that involve G-protein activation that do not produce active G-protein subunits that modulate the VDCC current directly. Instead, the activation of G-proteins results in the activation of enzymes, catalysing the formation of a number of active compounds that subsequently have a diverse array of effects, including modulation of VDCC currents. Amongst these second messenger pathways are the second messengers cyclic adenosine monophosphate (cAMP) and diacylglycerol (DAG) that activate protein kinase A (PKA) and protein kinase C (PKC) respectively, discussed below. The second messenger cascades are common signal transduction pathways used throughout cell cycles, providing amplification and several points at which the signal can be controlled or shaped.

Protein Kinase A (PKA)

Stimulation of β-adrenergic receptors results in increased levels of intracellular second messenger cAMP, the activator of cAMP-dependent protein kinase-A (PKA). At the G-protein level, this cascade involves the activation of $G_{\alpha}$ which activates adenylyl cyclase, resulting in the catalysis of the conversion of ATP to cAMP. The classical VDCC modulation provided by this cascade is the enhancement of cardiac L-type currents by norepinephrine (Reuter, 1983). The PKA phosphorylation sites appear to be present in the C-termini of VDCC $\alpha_{1S}$ (Rotman et al., 1995), $\alpha_{1C}$ (De Jongh et al., 1996; Gao et al.,
1997) and α1D (Mitterdorfer et al., 1996) (see Figure 1.4). VDCC modulation may also involve PKA phosphorylation sites located on auxiliary β subunits (Haase et al., 1993; Haase et al., 1996; Gao et al., 1997). The effect of cAMP-dependent PKA phosphorylation was investigated in single channel recordings and the current enhancement was shown to be due to an increase in the probability of opening and an extended duration for the channel opening. An opposing effect to phosphorylation-related current enhancement occurs when acetylcholine activates muscarinic receptors. Muscarinic receptor activation elicits an inhibition of cardiac calcium channels due to a reduction in the current enhancement arising from phosphorylation; the decreased rates of phosphorylation are due to inhibition of adenylyl cyclase and hence reduced cAMP levels, by the activated Gαi subunit (Jurevicius & Fischmeister, 1996).

There is a heterogeneous response in experiments attempting to reconstitute native cardiac L-type channel phosphorylation and the associated current modulation. Heterologous expression of α1C channels in several studies did not detect L-type current enhancement during PKA activating conditions (Perez-Reyes et al., 1994; Singer Lahat et al., 1994; Zong et al., 1995a; Perets et al., 1996). Some of these studies did suggest evidence for basal levels of channels phosphorylation, observing decreased calcium channel current amplitudes on application of kinase inhibitors (Singer Lahat et al., 1994; Perets et al., 1996). In other studies PKA activation did produce an obvious enhancement of L-type currents (Yoshida et al., 1992; Yatani et al., 1995). One explanation that was initially suggested for differences in PKA-dependent modulation of neuronal α1C was the observation that a long splice variant had the ability to be modulated by PKA, whilst the short splice variant (with a truncated C-terminus) did not exhibit modulation by PKA (Hell et al., 1993). More recently, an alternative explanation for the heterogeneous PKA modulation of L-type calcium channel currents has been suggested. A-kinase anchoring proteins (AKAPs) are thought to anchor the PKA proteins to the membrane, localising the phosphorylating protein in close proximity to its target proteins, such as VDCCs. PKA
phosphorylation of heterologously expressed α1S (Johnson et al., 1997) and α1C channels (Gao et al., 1997) were both shown to require the presence of AKAPs.

In addition to PKA phosphorylation of L-type VDCC by activation of GPCRs, an alternative PKA activation pathway has been suggested. A voltage-dependent PKA phosphorylation of L-type currents resulting in a current facilitation has been observed in bovine chromaffin cells (Artalejo et al., 1990), neuronal α1C channels expressed in oocytes (Bourinet et al., 1994) and cardiac α1C channels expressed in CHO cells (Sculptoreanu et al., 1993) in response to large depolarising pre-pulses. However these findings have been questioned by later studies: Albillos et al. (1996) state that the pre-pulse facilitation observed in chromaffin cells appears to be more consistent with a voltage-dependent autocrine feedback loop, and that previous studies were confused by autocrine secretions during flow-stop and superfusion; in other studies, heterologous expression of cardiac α1C channels did exhibit a form of pre-pulse facilitation, though the facilitation observed was intrinsic to an α1C/β subunit complex, and was unaffected by PKA phosphorylation (Dai et al., 1999; Kamp et al., 2000).

Though the L-type currents display the most prominent modulation by cAMP-dependent PKA phosphorylation, and have also been studied most extensively, other VDCC current types have been shown to exhibit modulation by this pathway. P-type channels expressed in oocytes have displayed current enhancement by elevating cAMP levels (Fournier et al., 1993). The N-type α1B subunit was shown to be phosphorylated by PKA (Ahlijanian et al., 1991), and in support of this observation a voltage-independent PKA phosphorylation was shown to be partly responsible for the selective inhibition of N-type currents in differentiated NG108-15 cells (Brown & Seabrook, 1995). In bass retinal cone cells, typical L-type enhancement was observed during cAMP-dependent PKA phosphorylating conditions, whilst simultaneously the same preparation was shown to inhibit the LVA T-type component of the current population (Pfeiffer-Linn & Lasater, 1993).
Protein Kinase C (PKC)

Like the effects observed upon cAMP-dependent PKA phosphorylation of L-type VDCC, PKC phosphorylation has been shown to enhance L-type currents in cardiac (Lacerda et al., 1988), smooth muscle (Schuhmann & Groschner, 1994) and neuronal tissues (Yang & Tsien, 1993). Studies of single-channel recordings showed that the enhancement was also due to an increased probability of channel opening (Yang & Tsien, 1993). However, unlike the PKA phosphorylation studies, in vitro reconstitution of the PKC phosphorylation effect on expressed cardiac α_{1C} channels was consistently observed by a number of different research groups (Bourinet et al., 1992; Singer-Lahat et al., 1992; Shistik et al., 1998). However, when similar experiments were performed in an attempt to observe PKC modulation of heterologously expressed neuronal α_{1C} channel currents no current modulation was forthcoming (Stea et al., 1995). In addition to the various L-type isoforms that exhibit PKC current enhancement, the N-type current also shows modulation by this phosphorylation pathway. Both enhancement (Yang & Tsien, 1993) and inhibition (Cox & Dunlap, 1992) of N-type currents have been observed. A series of N-termini truncation mutants of the α_{1C} channel have been performed, and show that the N-terminus is critical for the PKC modulation of these channels (Shistik et al., 1998). However, the N-terminus was not phosphorylated by PKC, and the site of PKC phosphorylation appears to be either a nearby N-terminus site or a closely associated auxiliary subunit (Shistik et al., 1999). The PKC phosphorylation pathway also provides cross-talk with G_{βγ} modulation of α_{1B} channels: a PKC site has been found on the α_{1B} I-II loop, which when phosphorylated by PKC results in an attenuation of G_{βγ} modulation of α_{1B} channel currents (Zamponi et al., 1997).

Additional kinases and second messengers that modulate VDCCs

Further to the modulation observed with PKA and PKC, there are a number of kinases that have been shown to exhibit modulation of VDCCs. Protein tyrosine kinases have
been shown to have enhancing and inhibiting modulatory effects on L-type calcium currents (Yokoshiki et al., 1996; Wang & Lipsius, 1998). Cyclic guanosine monophosphate (cGMP)-dependent protein kinase (PKG) was suggested to phosphorylate neuronal $\alpha_{1C}$ channels (Hell et al., 1993), and, further to this postulated pathway, neuronal $\alpha_{1C}$ channels were shown to be phosphorylated resulting in current inhibition by somatostatin activated PKG (Meriney et al., 1994). Recently the cardiac $\alpha_{1C}$ channel current was also shown to be inhibited by PKG, and the point of phosphorylation was determined to be on the $\alpha_{1C}$ subunit at Ser$^{533}$ (rabbit cardiac $\alpha_{1C}$ numbering; Jiang et al., 2000). Since the second messenger nitric oxide (NO) plays an important role in the regulation of cGMP formation, by activating guanylate cyclase, the close association between NO and PKG may often blur the boundaries between the two possible pathways, and NO may actually be behaving as a co-factor for the efficient activation of the PKG pathway (e.g. see Sperelakis et al., 1994; Xiong et al., 1994). The calcium-dependent calmodulin kinase II (CaM kinase II) has also been shown to phosphorylate $\alpha_{1C}$ (Benovic & Gomez, 1993) and also a long splice variant of $\alpha_{1B}$ (Hell et al., 1994). Oxygen in arterial myocytes may also behave as a second messenger, since low oxygen tension has been shown to inhibit L-type calcium channel currents (Franco-Obregón et al., 1995).

This discussion does not cover all the possible kinases and second messengers some of which are yet to be determined. For instance, Mathie et al. (1992) showed that a second messenger was responsible for oxotremorine-induced inhibition of L- and N-type VDCC, though the effect was not attributable to Ca$^{2+}$, cGMP, cAMP or PKC.

**Dephosphorylation of VDCCs**

Based on the number of kinases and potential phosphorylation sites found on the various VDCC subunits, it is no surprise that dephosphorylation also has a modulatory role upon VDCC currents. Numerous studies have shown that activation of phosphatases 1, 2A and 2B (also called calcineurin) and inhibition by their inhibitors (e.g. okadaic acid) modulated both L-type and non L-type currents (usually dephosphorylating channels
resulted in inhibition, reversing the enhancement produced by PKA, for example). A comprehensive discussion of the dephosphorylation effects on different VDCCs (and other ion channels) was written recently by Herzig and Neumann (2000), and will not be discussed further here.

1.4.4 VDCC interaction with other proteins

A number of proteins are closely associate with VDCCs in order to efficiently couple the Ca\(^{2+}\) that enters through the channel with Ca\(^{2+}\)-dependent processes that these proteins elicit.

**SNARE proteins (syntaxin and SNAP-25)**

The exocytosis of neurotransmitters is dependent on Ca\(^{2+}\), and therefore some of the exocytotic proteins directly interact with VDCC. The SNARE (soluble NSF attachment protein receptor) complex is a key component of the exocytotic protein machinery and is formed of synaptobrevin (also known as VAMP), syntaxin and synaptosome-associated protein of 25 kDa (SNAP-25)(Sollner *et al.*, 1993). Syntaxin and SNAP-25 have been shown to directly interact with a sequence located on the intracellular loop connecting domains II and III (II-III loop; see Figure 1.4) on the \(\alpha_{1A}\) (Rettig *et al.*, 1996) and the \(\alpha_{1B}\) VDCC subunits (Sheng *et al.*, 1994). This synaptic protein interaction site on the VDCC has been dubbed the synprint. Peptide blocking sequences targeted at the synprint have been shown to disrupt the Ca\(^{2+}\)-dependent release of neurotransmitters by N-type VDCCs (Rettig *et al.*, 1997). Intriguingly, syntaxin has also been shown to modulate the interaction between \(G_{\beta\gamma}\) and VDCCs (Stanley & Mirotznik, 1997), with the presence of syntaxin 1A optimising the \(G_{\beta\gamma}\) inhibition of \(\alpha_{1B}\) expressed channels (Jarvis *et al.*, 2000). This syntaxin/\(G_{\beta\gamma}\)/VDCC interaction provides a significant link between the processes of \(G_{\beta\gamma}\) inhibition of VDCCs and their role in neurotransmitter release. In addition to affecting the release of neurotransmitters and the modulation of \(G_{\beta\gamma}\) inhibition of \(\alpha_{1B}\) channels, syntaxin has been shown to slow the rate of channel of inactivation of
expressed $\alpha_{1A}$ and $\alpha_{1B}$ channels (Bezprozvanny et al., 1995; Wiser et al., 1996b). A recent study determined, using oocyte expression of $\alpha_{1B}$ channels, that this decrease in inactivation rate by syntaxin 1A was specifically observed in the slow, voltage-independent form of inactivation (Degtiar et al., 2000). Until recently interactions were only shown in the non L-type $\alpha_{1A}$ and $\alpha_{1B}$ channels. However, particularly pertinent to this study was the observation that syntaxin 1 not only co-localised with the $\alpha_{1D}$ L-type channels but also exhibited a functional effect upon the $\alpha_{1D}$ current, with syntaxin 1 presence reducing the Ca$^{2+}$-dependent run-down of $\alpha_{1D}$ currents (Yang et al., 1999). This study was performed in $\beta$-pancreatic cells, and the presence of functional syntaxin 1 was also shown to aid the exocytosis of insulin from these cells, providing a compelling link to $\alpha_{1D}$ VDCC currents and exocytosis/neurosecretion in specialised cells.

An interaction between the vesicle SNARE synaptotagmin and $\alpha_{1A}$ and $\alpha_{1B}$ subunits has also been observed, again interacting with the synprint sequence on the II-III loops of the two $\alpha_1$ subunits (Charvin et al., 1997; Sheng et al., 1997). Due to the overlapping binding of the SNARE proteins syntaxin and SNAP-25 with the $\alpha_1$ VDCC subunits and with synaptotagmin binding it was suggested that competitive binding between these proteins allows temporal-spatial control of the neurotransmitter release. Thus, a sequential series of protein bindings and unbindings all orchestrated with the entry of Ca$^{2+}$ via the VDCCs results in neurotransmitter release (see review by Catterall, 1999).

The ryanodine receptor (RyR)

The close proximity of VDCC Ca$^{2+}$ entry with a Ca$^{2+}$-dependent effector is also utilised by the process of calcium-induced calcium release (CICR; see also section 1.2.1). The Ca$^{2+}$-dependent effector is the ryanodine receptor (RyR) located in the membranes of the endoplasmic and sarcoplasmic reticulum. Excitation-coupling observed in smooth muscle (but not cardiac muscle) requires direct coupling between the $\alpha_{1S}$ VDCC and the RyR-1
(and conversely, no direct coupling is observed between cardiac $\alpha_{1c}$ and RyR-2). Channel chimaeras and peptide binding studies have shown that two points (see Figure 1.4) on the $\alpha_{1s}$ subunit are pertinent to the direct interaction and functional coupling with the RyR-1: the intracellular II-III loop (Tanabe et al., 1990; el-Hayek et al., 1995; Nakai et al., 1998; Grabner et al., 1999) and the C-terminus (Slavik et al., 1997) were shown to be important in this $\alpha_{1s}$ subunit interaction.

**Calmodulin (CaM)**

Calmodulin (CaM) is another protein that directly interacts with the $\alpha_1$ VDCC subunit. As was mentioned previously, when discussing Ca$^{2+}$-dependent inactivation (see section 1.3.3), a series of recent investigations have shown that CaM binds to the C-terminus of $\alpha_{1c}$ L-type channels in a Ca$^{2+}$-dependent manner, resulting in Ca$^{2+}$-dependent inactivation (Peterson et al., 1999; Qin et al., 1999; Zühlke et al., 1999; reviewed by Levitan, 1999). These studies showed that CaM binds to an IQ motif in the $\alpha_{1c}$ C-terminus, and a sequence alignment with the human $\alpha_{1d}$ isoform (Genbank No. m76558; used in this study) would suggest that this sequence is also responsible for the similar Ca$^{2+}$-dependent inactivation observed in the $\alpha_{1d}$ L-type channels:

\[
\begin{align*}
\alpha_{1c} & \text{ - YATFLIQeYFRKFKRKEQG - } \\
\alpha_{1d} & \text{ - YATFLIQdYFRKFKRKEQG - }
\end{align*}
\]

The residues specific to the CaM IQ binding motif are shaded in grey, though even in the nearby surrounding residues there is only a single residue difference between the $\alpha_{1c}$ and $\alpha_{1d}$ sequences. Analogous IQ sequences, though different within the critical grey shaded areas are found in the non L-type HVA VDCC $\alpha_1$ subunits ($\alpha_{1a}$, $\alpha_{1b}$ and $\alpha_{1e}$), and were shown to interact with CaM (Peterson et al., 1999), though CaM signal transduction (Ca$^{2+}$-dependent inactivation) is weak or absent in these $\alpha_1$ isoforms. Thus, Ca$^{2+}$-dependent CaM binding in the L-type channels appears to arise through a very specific IQ.
binding motif, and possibly additional sequences may be required for the complete inactivation mechanism to be elicited.

Considering the $\alpha_{1A}$ VDCC isoform has been shown to be only weakly inactivated by $\text{Ca}^{2+}$, a recent study defining $\text{Ca}^{2+}$-dependent inactivation site in $\alpha_{1A}$ channels was unexpected (Lee et al., 1999b). As observed for the L-type channels, this study again showed that the $\alpha_{1A}$ CaM-binding domain ($\alpha_{1A}$ CBD) lies in the C-terminus of the $\alpha_{1A}$ subunit and binds to CaM in a $\text{Ca}^{2+}$-dependent manner, but the binding motif was different from the IQ motif found in L-type channels. Blocking of the $\alpha_{1A}$ CBD with specific peptides, deletion mutants with the $\alpha_{1A}$ CBD removed or alteration of $[\text{Ca}^{2+}]_i$ by $\text{Ca}^{2+}$-chelators or $\text{Ca}^{2+}/\text{Ba}^{2+}$ substitutions all prevented the subtle but significant $\text{Ca}^{2+}$-dependent inactivation observed in expressed $\alpha_{1A}$ channels.

### 1.5 VDCC channelopathies

With increasing knowledge on the genetic aspects of VDCC subunits, a number of neurological and muscular disorders have been shown to have associations with VDCC subunits (see reviews by Burgess and Noebels, 1998; Jen, 1999). Many of these disorders involve genetic mutations, with mutations in the $\alpha_{1A}$ VDCC subunit the most studied so far. For example, mutations in $\alpha_{1A}$ have been attributed to the cause of some neurological disorders, including: familial hemiplegic migraine (Ophoff et al., 1996), episodic ataxia type 2 (Ophoff et al., 1996) and spinocerebellar ataxia type 6 (Zhuchenko et al., 1997). Mutations in $\alpha_{1S}$ have been shown to cause some muscular disorders including hypokalaemic periodic paralysis (Ptacek et al., 1994) and a mouse model for muscular dysgenesis (Chaudhari, 1992). The VDCC auxiliary subunits, too, have been implicated in genetic disorders: for instance, the $\beta_4$ subunit has been linked to the lethargic phenotype mouse model (Burgess et al., 1997) and the absence epilepsy mouse model (called *stargazer*) was found to be due to a mutation in the novel $\gamma_2$ subunit (see also section 1.3.4; Letts et al., 1998).
In addition to genetic mutations causing channel defects and subsequent diseased states, a number of pathologies exist which are associated with VDCCs but not due to genetic mutations. Lambert-Eaton myasthenic syndrome is an autoimmune disease in which auto-antibodies are raised against the \(\alpha_{1A}\) and \(\alpha_{1B}\) VDCC subunits (Lennon et al., 1995). In rats used as a model for non-insulin-dependent diabetes mellitus (Zucker diabetic rats) a reduced L-type current activity in pancreatic \(\beta\)-cells was shown to be caused by reduced \(\alpha_{1C}\) and \(\alpha_{1D}\) mRNA levels in these rat models (Roe et al., 1996).

1.6 Mammalian cell lines used

In this study three mammalian cell lines were used to investigate aspects of \(G_{\beta_\gamma}\) modulation of L- and N-type VDCC currents: the growth-hormone secreting cell line, \(GH_4C_1\); the human embryonic kidney (HEK) 293 cell line; and the COS-7 cell line. Each of these cell lines have been extensively investigated by numerous other research groups, including many studies specifically investigating G-protein effects on VDCC, and have therefore been shown to contain a range of active G-protein coupled pathways.

\(GH_4C_1\) cells

The \(GH_4C_1\) cell line is a clonal strain of a group of rat pituitary tumour cell lines including the \(GH_1\) and \(GH_3\) cell lines (Sonnenschein et al., 1970). The \(GH_4C_1\) cell line was derived from the \(GH_3\) cell line, and was initially chosen because the \(GH_4C_1\) cell line was shown to synthesise prolactin and growth hormone at greater rates (Tashjian, 1979). The \(GH_4C_1\) cells express a number of endogenous ion channels (Dubinsky & Oxford, 1984), including VDCCs which include both LVA (T-type) and HVA (predominated by DHP-sensitive L-type, >80%, with lesser fractions of N- and P-type currents)(Cohen & McCarthy, 1987; Seabrook et al., 1994). The predominantly L-type HVA VDCC have been shown to be functionally coupled and inhibited by activation of both endogenous sst2 and M4 receptors (Liu et al., 1994) and exogenously expressed human D2s
(Seabrook et al., 1994). Therefore, the GH4C1 cells provide a useful cell line in which G-protein modulation of neuroendocrine L-type currents can be investigated.

**HEK 293 cells**

The HEK 293 cell line is another useful expression system used for investigating VDCCs and G-protein modulation. It is generally accepted that HEK 293 cells do not endogenously express functional VDCCs (but see Berjukow et al., 1996), and therefore allow specific channel compositions to be exogenously expressed by transfection. HEK 293 cells have been shown to express endogenous sst2 receptors (Law et al., 1993), which when activated functionally couple and inhibit exogenously expressed VDCC currents (Toth et al., 1996). Endogenous G-proteins were also shown to couple to expressed VDCCs in HEK 293 cells by activation or preventing activation by dialysis with GTP-γS or GDP-βS, respectively (Meza & Adams, 1998). In addition a number of exogenous G-protein pathways have also been shown to couple to and inhibit expressed VDCCs in HEK 293 cells, for example: exogenous expression of group I metabotropic receptors (McCool et al., 1998) and muscarinic M2 receptors (Colecraft et al., 2000); and, overexpression of exogenous Gβγ subunits (Shekter et al., 1997).

**COS-7 cells**

The COS-7 cell line is a simian (African green monkey) kidney fibroblast cell line which, like the HEK 293 cells, has been used extensively in the investigation of VDCCs due to a lack of any endogenously functionally expressed VDCCs. Within Prof. Dolphin's laboratory the COS-7 cells expressing exogenous VDCCs have been investigated thoroughly and the cell line has a high propensity to successful transient expression of VDCC and G-protein subunit cDNA expression (e.g. see Dolphin et al., 1999). Throughout these studies endogenous currents have not been observed. Endogenous mRNA transcripts for VDCC β subunits have been demonstrated by RT-PCR, though subsequent immunocytochemistry did not reveal any β subunit protein within COS-7
cells (Meir et al., 2000). Once again, like the HEK 293 cells, G-protein modulating paradigms have been shown to be functionally coupled to exogenously expressed VDCCs in the COS-7 cells, including: exogenous $G_{\beta}$, over-expression; decreased levels of free $G_{\beta}$, by binding to expressed $\beta$-adrenergic receptor kinase C-termini; and, modulation by dialysis of GTP-$\gamma$S or GDP-$\beta$S (Page et al., 1997; Page et al., 1998; Meir & Dolphin, 1998; Stephens et al., 1998b; Canti et al., 1999).
Epilogue to Introduction

As research proceeds it is becoming apparent that the modulation of VDCCs involves a complex web of associated proteins. The auxiliary VDCC subunits were obvious candidates for initial sources of channel modulation, but increasingly subtle interactions are also now being discovered, with a vast array of modulatory mechanisms available. G-proteins, kinases, phosphatases and other proteins and second messengers all directly bind and shape the VDCC current. Predictably, with such close interactions between several proteins at the VDCC focal point, further complexity of VDCC modulation has been shown with cross-talk between modulatory pathways. For instance, PKC phosphorylation modulates both the $\alpha_1$ VDCC biophysics directly and also reduces $G_{\beta y}$ binding to the $\alpha_1$ VDCC subunit; syntaxin, too, has been shown to affect channel kinetics and additionally optimise $G_{\beta y}$ binding to the $\alpha_1$ VDCC subunit. The interactions between all these VDCC associated proteins is likely to continue growing, and with it an increasing complexity and subtlety of VDCC modulation and VDCC actions are highly probable. In initial studies upon exocytotic machinery proteins, the SNARE protein complex was discovered as a core of three proteins and has subsequently been shown to be a complex architecture of up to 20 interacting proteins (or more with complicated exocytosis models; see review by Mochida, 2000). In an analogous scenario to that observed with the exocytotic protein machinery, it is likely that the future VDCC complex will be envisaged as a core-complex formed of a combination of the VDCC subunits ($\alpha_1$, $\alpha_2$, $\beta$, and/or $\gamma$) associated with an interaction hierarchy of supporting and modulating proteins completing the overall VDCC structure.

It is the interaction between the neuroendocrine ($\alpha_{1D}$) L-type channel and activated $G_{\beta y}$ protein, and the effects of VDCC $\beta$ subunit co-expression on $G_{\beta y}$ modulation of $\alpha_{1B}$ that has been investigated in this study, providing information on a specific set of interactions within the increasingly complicated VDCC architecture.
AIMS

There is still uncertainty regarding the molecular derivation of the neuroendocrine L-type current that has been shown to exhibit fast, membrane-delimited and voltage-dependent G-protein modulation in several studies (Haws et al., 1993; Tallent et al., 1996; Piros et al., 1996; Degtiar et al., 1997). The principal aim of this study was to investigate the molecular nature of this neuroendocrine L-type current using whole-cell and perforated patch clamp recording techniques. Two cell lines were used to investigate G-protein modulation of neuroendocrine L-type currents: rat pituitary GH\textsubscript{4}C\textsubscript{1} cells stably expressing the human D2\textsubscript{S} dopamine GPCR (GH\textsubscript{4}C\textsubscript{1} D2 cells) and HEK 293 cells stably expressing a neuronally modelled VDCC subunit complex (\(\alpha_{ID}\), \(\alpha_{2}\delta-1\) and \(\beta_{3}\); HEK 293 \(\alpha_{ID}\) cells).

Using the GH\textsubscript{4}C\textsubscript{1} D2 cells, pilot investigations were performed to determine practical intracellular recording solutions, some basic channel biophysics and to study the presence of G-protein modulation of the predominant L-type currents expressed in these cells. The HEK 293 \(\alpha_{ID}\) cells were another target cell line in which an aim was to pharmacologically and biophysically characterise the neuronal L-type current, and follow these characterisation experiments with further experiments investigating the G-protein modulation of the current. Thus, the investigation of the currents in these two cell lines would attempt to clarify possible VDCC subunit sources of the neuroendocrine L-type current that had previously been shown to exhibit G-protein modulation in a number of cell types.

The complexity of protein interactions associated with VDCCs, particularly with reference to G-protein interactions, was further investigated in a second study objective. The auxiliary \(\beta\) VDCC subunit has been shown to have a modulatory effect upon G-protein inhibition of VDCC currents. Single-channel recordings made by Meir et al. (2000) had shown that G-protein modulation of \(\alpha_{1B}\) channels required the presence of VDCC \(\beta\) subunits in order to observe characteristic G-protein modulation of the current.
This result was unexpected since most previous investigations suggested the opposite, with VDCC \( \beta \) subunit acting antagonistically to the G-protein modulation of VDCC currents. Thus, a second proposed area of study was to investigate the effect of VDCC \( \beta \) subunit co-expression upon the G-protein modulation of \( \alpha_{1B} \) channels. To clarify the single channel observations made by Meir et al. (2000) in COS-7 cells, whole-cell patch clamp recordings were made with COS-7 cells transfected with \( \alpha_{1B} \) with or without \( \beta_{2a} \) subunit cDNA. Using co-expression of the rat dopamine D2L receptor to activate \( G_{\beta\gamma} \) subunits the G-protein modulation of the \( \alpha_{1B} \) channels formed with and without \( \beta_{2a} \) could then be studied, allowing a more definitive interpretation of the single-channel recording data.
CHAPTER 2

METHODS
2.1 Materials

2.1.1 Compounds

The following compounds were stored as stocks at -20°C (concentration in mM unless stated, solvent and source): nifedipine, NIF (3, ethanol, Sigma, St. Louis, MO, USA); nicardipine, NIC (3, ethanol, Sigma); S-(-)BayK8644, BayK (3, ethanol, RBI, Natwick, MA, USA); somatostatin-14, SS-14 (0.1, deoxygenated double-distilled water, RBI); quinpirole, Quin (10, double-distilled water, RBI); dopamine, DA (10, double-distilled water, RBI); carbachol, Carb (100, double-distilled water, Sigma); forskolin (10, dimethyl sulphoxide, Sigma); ω-conotoxin GVIA, ω-CgTx GVIA (0.1, deoxygenated double-distilled water, Alomone Labs, Jerusalem, Israel); geneticin G-418 sulphate, G-418 (100 mg.ml⁻¹, double distilled water, Life Technologies, Paisley, Scotland); Zeocin (100 mg.ml⁻¹, supplied in solution form, Invitrogen, Carlsbad, CA, USA); nystatin (50 mg.ml⁻¹, dimethyl sulphoxide, Sigma); and amphotericin-B (80 mg.ml⁻¹, dimethyl sulphoxide, Sigma).

2.1.2 cDNA

The following cDNAs were used in transient transfections: rabbit α₁B (GenBank accession number D14157), rat α₁E (rbEII, L15453), rat α₁Elong, rat β₁b (Tomlinson et al., 1993), rat β₂a (M80545), rat β₃ (M88571), rat α₂S-1 (neuronal splice variant, M86621), rat D₂long receptor (rD₂L, X17458, N5→G), and mut-3 green fluorescent protein (mut-3 GFP, U73901).

In transient transfections involving α₁E and those carried out by electroporation in COS-7 cells, cDNAs were subcloned into the expression vector pMT2 (Genetic Institute, Cambridge, MA, USA), see Swick et al., 1992. COS-7 cells are SV-40 transformed cells, and therefore constitutively express the SV-40 T antigen. The presence of the SV-40 T antigen allows high replication efficiency of the pMT2 vector (including the inserted calcium channel subunit cDNA) via a SV-40 origin of replication found within pMT2 (for further explanation see Sambrook et al., 1989). The adenovirus major late promoter controls the
expression of inserted cDNA in pMT2. Attempts to express calcium channel subunits using pMT2 in HEK 293 cells proved fruitless: efficient expression using this vector was never attained (data not shown). HEK 293 cells do not express the SV-40 T antigen (unlike the SV-40 transformed COS-7 cells), limiting the efficiency of pMT2 expression in HEK 293 cells. Therefore an alternative vector was used for transient expression of HEK 293 and the HEK 293 α1D cell lines: all the cDNAs were subcloned into the pRK5 expression vector. The pRK5 vector expresses inserted cDNA under the control of the cyto-megalovirus promoter, a promoter that appears to efficiently express inserted cDNA in HEK 293 cells. The subcloning of channel subunits and receptors into pMT2 and pRK5 were performed using standard molecular biology techniques (excision of required clone via restriction enzymes and subsequent ligation into the complementary restriction digested vector, see Sambrook et al., 1989) by Drs. K. Page and N. Berrow.

The rat α1E (rbEII, L15453) clone has a truncated N-terminus. Page et al. (1998) extended this clone using a rat α1E N-terminal extension (AF057029), cloned from rat cerebellar granule cells. The resulting rat α1Elong clone has homology to RT-PCR products from published mouse (L29346), human (L27745) and rabbit (X67855) α1E clones. This rat α1Elong clone was used in this study.

The β1b subunit used in this study is that of Tomlinson et al. (1993). It is identical to the rat β1b clone defined in the GenBank database (X61394) except for two substitutions (R417→S and V435→A) and the deletion A431 (T. Snutch, personal communication).

2.2 Creation of stable cell lines

2.2.1 GH4C1 stably transfected with huD2s

The GH4C1 cell line was stably transfected with the short isoform of the human dopamine D2 (huD2s) receptor by Dr. Guy Seabrook (Merck Sharp & Dohme) according to methods described in McAllister et al. (1993) and Seabrook et al. (1994); the resulting huD2s
expressing cell line will subsequently be referred to as the GH4C1 D2 cell line. The huD2 short receptor was cloned by PCR from human brain mRNA (Clonetech Inc., Palo Alto, CA, USA), and restricted digested with EcoRI and XhoI and subsequently subcloned into the expression vector pcDNA1neo (Invitrogen, Carlsbad, CA, USA). The huD2s-pcDNA1neo vector was then transfected into GH4C1 cells using the Ca²⁺ phosphate precipitation transfection protocol. Stably expressing clones were selected via G-418 selection pressure. The presence of the huD2s receptor was confirmed by [¹²⁵I]-iodosulpiride or [³H]-spiperone binding assays and PCR analysis.

2.2.2 HEK 293 α₁D stable cell line

Standard molecular biology was used to stably transfect HEK 293 with human neuronal α₁D (M76558), α₂βδ-1 (M76559) and β₃a (not published). For the cloning of these VDCC subunits see Williams et al. (1992b). The subsequent establishment of the clonal α₁D cell line is essentially as described in Williams et al. (1992a), and was performed by these researchers at Merck Research Laboratories (MRL), CA, USA. Briefly, HEK 293 cells were transfected using a standard Ca²⁺ phosphate procedure (Brust et al., 1993) with 10, 5 and 5 μg of the α₁D, α₂βδ-1 and β₃a expression constructs, respectively. The α₁D subunit expression plasmid, pcDNA1α₁D RBS, does not contain an antibiotic resistance gene, whereas the α₂βδ and β₃a subunit expression plasmids, pRcCMVα₂βδ-1 and pZeoCMVβ₃a, contain the neomycin and Zeocin resistance genes, respectively. G-418 (final concentration 100 μg.ml⁻¹, Life Technologies, Paisley, Scotland) and Zeocin (final concentration 40 μg.ml⁻¹, Invitrogen) antibiotics were used for selection of colonies. Selection medium was added to the cells 48 h after transfection. Antibiotic resistant colonies were transferred to 96-well plates using cloning cylinders two to four weeks after selection was initiated. Cell lines containing functional channels were selected with a fluo3-based calcium flux assay. Selection pressure of the HEK 293 α₁D cells was maintained throughout culturing by addition of the anti-biotics G-418 and Zeocin (at the concentrations described above).
2.3 Cell culture

2.3.1 GH<sub>4</sub>C<sub>1</sub> D2 cell culture

The GH<sub>4</sub>C<sub>1</sub> D2 cell line used was provided by Dr. G. Seabrook (Merck Sharp & Dohme, Harlow, UK) and grown according to protocols found in Seabrook et al., 1994. Briefly, cells were grown in Dulbecco’s Modified Eagle’s Medium (GIBCO), supplemented with 10% foetal calf serum (GIBCO), 1% penicillin-streptomycin (10 000 IU.ml<sup>-1</sup>, penicillin, 100 μg.ml<sup>-1</sup>, GIBCO) and 8 mM L-glutamine (Sigma). In addition during the first 2-3 passages after reviving from liquid nitrogen storage stocks were grown in 0.6 mg.ml<sup>-1</sup> G-418. Cells were enzymatically dissociated from 25 cm<sup>2</sup> flasks (Becton & Dickinson) using trypsin (0.5 mg.ml<sup>-1</sup>)-ethylenediaminetetraacetic acid (EDTA; 0.5 mM in Puck’s saline; GIBCO) applied for 2 min at room temperature. Approximately 1-2x10<sup>5</sup> cells.ml<sup>-1</sup> were plated on poly-L lysine hydrobromide coated (MW 150-300,000; 15 μg.ml<sup>-1</sup>; Sigma) cover slips, and recordings were made 4-5 days after plating.

2.3.2 Culture of HEK 293 and HEK 293 α<sub>ID</sub> cell lines

The culture medium in which the stable HEK 293 α<sub>ID</sub> (obtained from Dr. K. Stauderman, MRL, CA, USA) and control HEK 293 cells (ATCC 1573) were grown, consisted of Dulbeccos Modified Eagle’s Medium with 4500 mg glucose (DMEM, Life Technologies). This was supplemented with 5% Defined Bovine Calf Serum (Hyclone, Utah), 1% penicillin-streptomycin (10 000 IU.ml<sup>-1</sup>, penicillin, 100 μg.ml<sup>-1</sup>, GIBCO) and the additional selection antibiotics for the HEK 293 α<sub>ID</sub> cell line (as described above, see 2.2.2). The cells were grown in this medium at 37°C, 5% CO<sub>2</sub> and humidity and passaged every 2-3 days. Enzymatic dissociation was used (as described for the GH<sub>4</sub>C<sub>1</sub> D2 cells, above) and dissociated cells were plated on poly-L lysine hydrobromide coated cover-slips at a current density of approximately 1-2x10<sup>5</sup> cells.ml<sup>-1</sup>. The cells were left to adhere to the cover-slips
for 6 h at 37°C, before being transferred to a 28°C incubator. Electrophysiological recordings were made from fragments of these cover-slips placed into the perfusion chamber after a further 36 h incubation.

2.3.3 COS-7 cell culture

COS-7 cells (obtained from the European Collection of Animal Cell Cultures) were grown and passaged in a similar fashion to that explained for HEK 293 cells. The growth medium used was Minimum Essential Medium (MEM; GIBCO) Alpha supplemented with 10% New Born Calf Serum (Life Technologies) and 1% penicillin-streptomycin (10 000 IU.ml⁻¹, penicillin, 100 μg.ml⁻¹, GIBCO).

2.4 Transient transfections

2.4.1 Transfection of HEK 293 with Geneporter reagent

For transient transfection of the α₁b, α₂δ, β₃a VDCC subunits and mut-3 GFP expression marker into HEK 293 cells, a mixture consisting of 15, 5, 5, and 1 μl (at a concentration of 1 μg.μl⁻¹; all in the pRK5 expression vector) of each cDNA species respectively was made. In experiments where the rD2₄ was used, 5 μg of this cDNA was added; in experiments where this D2 receptor pathway was not investigated, 5 μg of blank pRK5 vector was used to give a final cDNA amount of 31 μg. The same amounts were used for the transfection of α₁E, α₂δ, β₁b and mut-3 GFP (using 5 μg of blank pMT2 to make the mixture up to a final amount of 31 μg). Cells were dissociated from growing flasks by trypsin-EDTA (Life Technologies) treatment and acutely isolated by trituration. 2 ml of cell suspension were plated onto 35 mm culture dishes at a concentration of approximately 1x10⁶ cells.ml⁻¹, and left to adhere for 2 h at 37°C. During this stage, 10 μl of Geneporter reagent (Genetic Therapy Systems, San Diego, CA, USA) and 2 μl of the cDNA mix are added to 1 ml of DMEM (no supplements) and left at room temperature for 1 h. This solution was then added to the adhered cells after
washing the cells with phosphate buffered saline. The cells and cDNA solution were then incubated at 37°C for 3-4 h before adding a further 1 ml of DMEM (supplemented with 20% BCS and anti-biotics). These transfected cells were then grown at 37°C for 36 h before being trypsin treated and re-plated onto poly-L lysine coated cover-slips (incubated at 28°C for 6 h, followed by 36 h at 37°C before patching, as was described above in section 2.3.2). In HEK 293 α1D cell line experiments where additional transient transfection of rD2L expression was required the cDNA mix was formed of rD2L (5 µg) and mut-3 GFP (1 µg) cDNA and made the mix made up to 31 µg with blank pRK5 vector, with the transfection procedure being as described above (for α1B and α1E). Successful transfection was determined by expression of mut3-GFP. The epifluorescence unit on the inverted microscope utilised a high-pressure mercury lamp (Hg100W, LH-M100CB-1 lamphouse; Nikon UK Ltd., Surrey, UK) with a fluorescein filter block (520 nm) and dichroic mirror (510 nm).

### 2.4.2 Transfection of COS-7 cells via electroporation

Approximately 6-7x10⁶ cells were washed with 3-4 ml phosphate buffered saline (PBS; from PBS tablets consisting: 0.01 M phosphate buffer, 0.0027 KCl, 0.137 M NaCl, pH 7.4; Sigma) and dissociated by trypsin-EDTA (1 ml) treatment of a confluent monolayer of COS-7 cells from a growth flask (75 cm², Becton and Dickinson). After addition of 6 ml of MEM Alpha medium, the total of 7 ml of cell suspension was centrifuged at 300 x g for 3 min at 4°C. The resulting pellet was then washed with 7 ml of PBS and re-pelleted as before, centrifuging for 3.5 min. The supernatant PBS wash was then removed and the pelleted cells were suspended in 400 µl of pre-heated (to 37°C) media. This cell suspension was then added to the plasmid cDNA in a 2 mm electroporation cuvette (Equibio, Kent, UK). The plasmid cDNA was a composition of the following (in µg): α1B (15), α2δ (5), β2a (5), rD2L (5) and mut-3 GFP (1). In experiments where no β subunit was required the β2a was replaced by 5 µg of blank pMT2 (to give a final quantity of 31 µg). This sample was electroporated at 260 V and 1050 µF for 28.5 ms using the Easyject Plus electroporator (Equibio). The
surviving cells were then resuspended in 30 ml of pre-heated (37 °C) MEM alpha, 15 ml of which was added to a 75 cm$^2$ growing flask. After further incubation and growth at 37 °C for 72 h, the cells were dissociated from the flask by trypsin-EDTA treatment, and plated onto cover-slips (as above). Cells were left to adhere to the cover-slips for 1 h at 37 °C before transferral to a 28 °C incubator. These cells were then patched for up to 24 h after this replating. Once again, successful cDNA expression was determined by GFP UV fluorescence.

2.5 Reverse Transcription Polymerase Chain Reaction (RT-PCR) of clonal cell lines

The RT-PCR technique allows very sensitive and specific amplification of cDNA in vitro, essentially reproducing the transcription and DNA amplification processes observed in vivo. The amplification of specific DNA targets indicates the presence of the corresponding mRNA species within samples. However, due to the additional post-transcriptional modifications and translational controls, mRNA transcripts do not necessarily indicate subsequent translation and functional protein expression; a caveat that must be kept in mind when interpreting RT-PCR data.

RT-PCR is based on the following steps: extraction and purification of mRNA; first strand cDNA synthesis (the reverse transcription step creating the cDNA template); and specific DNA target sequence amplification (the polymerase chain reaction step). Throughout the PCR procedures hands were gloved, double-distilled and sterilised water was used and a level of sterility was maintained in order to prevent RNase or foreign DNA from contaminating the reaction mixtures.

2.5.1 mRNA preparation

Approximately 3-6x10$^6$ cells were removed from growing flasks by trypsin-EDTA treatment (see section 2.3.1). mRNA extraction and purification from these cells was carried out

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according to a standard guanidium isothiocyanate (GITC) method (Chomczynski & Sacchi, 1987). In brief: the cells were pelleted by centrifugation at 300 x g for 3 min at 4 °C, resuspended in 1 ml of HEPES buffered saline solution (HBSS) and re-pelleted in a sterile eppendorf tube (centrifugation as before). The pellet was homogenised in 500 μl of GITC solution (consisting of: GITC, 50 g; sodium citrate, 0.75 M, pH 7, 3.5 ml; N-lauroyl sarcosinate, 10%, 5.3 ml; mixed and made up to 106 ml with distilled water; freshly supplemented with 7 μl of 2-mercaptoethanol). The GITC (a strong denaturing agent) and mercaptoethanol (a reducing agent) effectively inactivate RNAses, thus preventing unwanted digestion of the required RNA. To this homogenate 50 μl of sodium acetate (2 M, pH 5) was added and mixed thoroughly (vortexed) for 5 s; subsequently, 500 μl of acidic phenol (Sigma) was added and vortexed for 10 s; 100 μl of chloroform was then added before being vortexed again for 10 s. The resulting mixture was then left to stand on ice for 10 min before being centrifuged at 13 000 x g (at 4 °C for 20 min). The upper, aqueous layer resulting from this centrifugation contains the required mRNA, and was removed to a fresh eppendorf tube and then vortexed with 300 μl phenol and 300 μl chloroform for 10 s. A second round of incubation on ice (5 min) and centrifugation (13 000 x g, 4 °C, 10 min) was then performed; again, the upper aqueous layer was removed from the centrifuged mixture and added to another fresh eppendorf tube. The mRNA contained in this aqueous layer was extracted by chloroform extraction: 300 μl chloroform was added, vortexed (10 s) before allowing chloroform and aqueous layers to reform (1-2 min at R.T.). The lower chloroform layer (used to extract remaining DNA and protein impurities) was then removed and discarded. To the remaining aqueous layer an equivalent volume (approximately 0.5 ml) of iso-propanol was added and incubated at −20 °C overnight. The precipitated RNA was then pelleted by centrifugation at 13 000 x g (4 °C, 20 min), washed with 70% ethanol (cooled to −20 °C), air dried and dissolved in 20μl of RNase-free water (double-distilled water treated with 0.1% diethylpyrocarbonate, DEPC, for >12 h, and autoclaved).
2.5.2 Concentration and purity of extracted mRNA

By using a spectrophotometer the quantity and purity of mRNA extracted from the cells was determined. A 5 µl sample of the mRNA was added to 595 µl of DEPC treated water (using 5 µl of sodium dodecyl sulphate in 595 µl of DEPC treated water as a negative control blank). From this dilute sample absorbance readings were taken at 260 nm and 280 nm wavelengths. At 260 nm an optical density (OD$_{260}$) of 1 is equivalent to 40 µg.ml$^{-1}$, therefore allowing calculation of the concentration of mRNA in the sample. The ratio of OD$_{260}$/OD$_{280}$ defines the sample purity: a ratio $<1.8$ is indicative of protein impurities, whilst a ratio $>2.0$ suggests sample degradation. Only samples giving OD$_{260}$/OD$_{280}$ ratios between 1.8-2.0 were used.

2.5.3 First strand cDNA synthesis by reverse transcription

This step involves the synthesis of cDNA from the extracted mRNA by reverse transcription (RT). The following reaction mixture was used for each of the cell types:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>dd H$_2$O</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>5xM-MLV RT Buffer</td>
<td>10 µl</td>
</tr>
<tr>
<td>dNTP (25 mM each)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Random Primers</td>
<td>2.5 µl (10pmol.µl$^{-1}$)</td>
</tr>
<tr>
<td>RNasin</td>
<td>1 µl (40u.µl$^{-1}$)</td>
</tr>
<tr>
<td>M-MLV RT</td>
<td>2.5 µl (200u.µl$^{-1}$)</td>
</tr>
<tr>
<td>RNA</td>
<td>30 µl</td>
</tr>
<tr>
<td>RNA</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

M-MLV RT (Moloney murine leukaemia virus reverse transcriptase; Promega, Madison, WI, USA) is the enzyme that reads the mRNA strand synthesising a cDNA strand alongside. This enzyme is supplied with a specific RT-buffer (Promega). In order to carry out this synthesis the M-MLV RT requires double stranded sections to prime with: therefore random primers (formed from random hexamers of bases, Promega) are added to provide priming sites on the mRNA, thus allowing the creation of cDNA copies of all of the mRNA species present. The mix also contains the cDNA building blocks, namely each of the four dNTPs (deoxyribonucleotide triphosphates, dATP, dTTP, dGTP and dCTP, Promega). In addition an RNase inhibitor (RNasin, Promega) is also included to prevent degradation of the mRNA.
during cDNA synthesis. This first strand synthesis mix was incubated at 37 °C for 2 h, and then diluted with double-distilled water to give a final volume of 100 µl. This was stored as 20 µl aliquots at -20 °C.

2.5.4 Determination of the presence of VDCC subunit cDNA by PCR

The thermal cycling of a polymerase chain reaction (PCR) mixture has three main components:

i. Denaturing of the cDNA at 95 °C. This creates single stranded cDNA allowing access of the specific oligonucleotide primers.

ii. Annealing of target cDNA with primers. This reaction occurs between 60-65 °C, is largely dependent on the target sequence G-C content and is predictable by primer design software.

iii. Polymerase extension, and hence creation of the amplified products, at 72-75 °C. This temperature range is optimal for the different DNA polymerase enzyme activities

Having created the first strand cDNA from mRNA extracted from each of the cell lines (see above), the specific amplification (PCR) of VDCC subunit cDNA was then performed. In essence, PCR of a target cDNA sequence requires: a thermo-stable DNA polymerase [such as PCR Taq polymerase (Promega), used in this study]; oligonucleotide primers (target-specific DNA sequences complementary to the designated sequence; designed by Drs. K. Page and N. Berrow, supplied by Perkin-Elmer, Foster City, CA, USA); and sufficient dNTPs for the amplification products.

For each PCR a master mixture composed of the following components was created:

- dd H2O: 17.5 µl
- 10x PCR Buffer (supplied with enzyme): 5 µl
- dNTP (10 mM each): 4 µl
- 25 mM MgCl2: 3 µl
- Taq Polymerase (5 U.µl⁻¹): 0.5 µl
- 30 µl

This use of master mix provides a simplified procedure, creates uniform PCR reaction mix for each of the PCR reaction tubes and limits the potential for sample contamination. This
master mix provides the components for a single PCR that is sufficient for amplification of a
specified VDCC subunit, and was therefore multiplied to give the amount required for
several PCR mixtures for each of the desired VDCCs. This master mix was then added to the
specific VDCC subunit forward (f) and reverse (r) primer pairs along with the clonal cell line
cDNA, in the following PCR reaction mix:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Master mix</td>
<td>30 µl</td>
</tr>
<tr>
<td>cDNA (from RT step)</td>
<td>5 µl</td>
</tr>
<tr>
<td>Primer f</td>
<td>10 pmol</td>
</tr>
<tr>
<td>Primer r</td>
<td>10 pmol</td>
</tr>
</tbody>
</table>

The PCR mix was then made up to 50 µl with double-distilled water. The primers created by
Perkin-Elmer were supplied at a given concentration (approximately 10 pmol.µl⁻¹) and thus
the amount of water to be added was calculated for each individual forward and reverse
primer pair.

**GH4C1 D2 cell line**

The specific forward and reverse primers and expected amplified fragment size (in base
pairs) for each of the VDCC subunits investigated in the rat GH4C1 D2 cell line are shown in
Table 2.1. For each VDCC amplification reaction a negative control (sterile, distilled water),
a positive template (1 µl of 100 pg.µl⁻¹ subunit specific cDNA created by Drs. K. Page and
N. Berrow; except for α1B for which the positive control rat dorsal root ganglion cDNA,
which has previously been shown to contain α1B mRNA, Regan et al., 1991) and the GH4C1
D2 cDNA test template. In addition an overall positive control for the ubiquitous cytosolic
protein actin was PCR amplified. The thermal cycle profile for the amplification was as
follows: 95 °C for 3 min (1 cycle); 95 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min (30
cycles); and finally, 72 °C for 5 min (1 cycle). The thermal cycle profile was performed
using a Touchdown thermal cycler (Hybaid, Middlesex, UK). PCR reaction mixtures are
routinely over-laid with mineral oil to prevent evaporation of the reaction mixture during
thermal cycling; however, this was not necessary since the thermal cycler used has a heated
lid. A 10 µl aliquot of the resulting amplification products were mixed with 5µl of loading
buffer [0.25% (w/v) bromophenol blue; 40% (w/v) sucrose; 0.1 M EDTA, pH 8.0; 0.5% (w/v) sodium lauryl sulphate (SDS)] loaded on a 2% (w/v) agarose gel, alongside a 100 base pair ladder (Amersham Pharmacia Biotech, Buckinghamshire, UK). The gel was made in tris-buffered acetate (TAE) that is stored as a 50 x concentrate (242 g of Tris base; 57.1 ml of glacial acetic acid; 100 ml of 0.5 M EDTA, pH 8.0; all from Sigma) and is diluted accordingly (with distilled water) on use. The loaded gel was then submersed in a TAE filled electrophoresis bath, electrophoresed, stained with ethidium bromide (0.01 ug.ml⁻¹, Sigma) and visualised by UV fluorescence.

Table 2.1 PCR primers for amplification of VDCC subunits in GH₄C₁ D2 cells.

<table>
<thead>
<tr>
<th>VDCC Subunit</th>
<th>5'→3' SEQUENCE</th>
<th>Amplification product size (bp)</th>
</tr>
</thead>
</table>
| α₁A          | f CAGCATCACAGACATCCTCG  
               | r AGACACGCAGCTACTCATCC  | 578 |
| α₁B (1)      | f CTGCTCGTCTGTGACAGTG  
               | r TTCTGGAGCCTTAGCTGACTG  | 1522 |
| α₁B (2)      | f CGAAATGACCTCATCCATGCAG  
               | r TTCTGGAGCCTTAGCTGACTG  | 1064 |
| α₁C          | f CGCAACACGTAGCTACTACCAG  
               | r GACCTAGAGAGCGAGCGAAGGA  | 574 |
| α₁D          | f CGTCAGCAATACGCAAGCAGCA  
               | r CGCTCCTACATTCCGTCCATT  | 754 |
| α₁E          | f CTGACAGCCTCCACACATCAT  
               | r AACACCTACAAGTCAGAGCAG  | 770 |
| β₁           | f CCTATGACGTGTTGCTCCGCC  
               | r CCTGGGCTTGGTGACAGA   | 883 |
| β₂           | f GTACCATCCATGAGACAGGT  
               | r TCAGTCCTGTCATGACAGAT  | 686 |
| β₃           | f CTCTAGCCAGACAGACAGAA  
               | r AGGCATCTTCTAGTCCTTC  | 831 |
| β₄           | f ATCAATGCGTCCTGTTGCTG  
               | r CAAGCGTTTCTACTCTCTG  | 772 |
| ACTIN        | f TTGTAACCAACTGGGACGCAT  
               | r GATCTTTCATGCTGTCTAGG  | 762 |
HEK 293, HEK 293 αID and COS-7 cell lines

Due to the similarity between the human and simian sequences for the chosen target VDCC subunit sequences it was possible to carry out PCR amplification of VDCC subunits in each of these cell lines using the same forward and reverse primer sets, shown in Table 2.2. It should be noted that no β2 was amplified. In COS-7 cells PCR amplification of the known LVA clones αIg, αIH and αII were additionally attempted. In order to search for all three clones degenerate forward and reverse primers were created: that is, in the forward primer G/C nucleotides can be interchangeable (where indicated), and in the reverse primer G/A can be interchangeable. Such degenerate primers were supplied as a mixture of all the possible different primers arising from this designated nucleotide degeneracy. A negative (distilled water), test sample (cDNA extracted from each specific cell line) and positive cDNA templates were used in each VDCC subunit amplification, as was described for GH4C1 cells (though for clarity only amplification products from the cDNA test template are shown in gels for HEK 293, HEK 293 αID and COS-7 cells). Almost identical thermal cycles were used for each of these three cell lines, and were as follows: 95 °C for 3 min (1 cycle); 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min (35 cycles for HEK 293 and HEK 293 αID cell lines; 30 cycles for the COS-7 cells); and finally 72 °C for 5 min (1 cycle). Electrophoresis and UV visualisation was as described for the GH4C1 D2 cell line. In addition to the 100 base pair marker ladder used for the GH4C1 D2 cells, a lamda BstE II digest ladder (Sigma) was also electrophoresed alongside amplification products for each of these cell lines.
Table 2.2  PCR primers for amplification of VDCC subunits in HEK 293, HEK 293 $\alpha_{1D}$ and COS-7 cells.

<table>
<thead>
<tr>
<th>VDCC Subunit</th>
<th>5'→3' SEQUENCE</th>
<th>Amplification product size (bp)</th>
</tr>
</thead>
</table>
| $\alpha_{1A}$ | f CCTCCTCTACGAGAAGAATG  
                 r AGACACGCACGTACTCATCC | 1256 |
| $\alpha_{1B}$ | f ACCCTGGAGCAGACACA  
                 r GGGCTTTATTCCGTCGCTTA | 1511 |
| $\alpha_{1C}$ | f ACCCTGGAGCAGACACA  
                 r GGGCTTTATTCCGTCGCTTA | 1698 |
| $\alpha_{1D}$ | f TTAGTGACGGCTGGAAACACG  
                 r GTGTTGTTCTTCCGCAAGGTA | 1116 |
| $\alpha_{1E}$ | f GAAGTCATCATGAAGGCCA  
                 r AGCAAGCATGACTTCCCTG | 551 |
| $\alpha_{1O/1H}$ | f GGCGTG/CATGC/AGAGA/ACTCT  
                 r GATGATGGTGGGG/ATATTGAT | 491 |
| $\alpha_{2\delta}$ | f GAACCTCAACTCGACAAGTGCCT  
                 r GCCATCCACTGAATACGTCCT | 664 |
| $\beta_1(i)$ | f CCTATGACGTGGTCCTTCC  
                 r TACATGGCATGTTCCTGCTC | 771 |
| $\beta_1(ii)$ | f AAATAAGCAAAGAGGABBGG  
                 r TCCATCGTGGTCCTGACT | 1414 |
| $\beta_3$  | f CTCTAGCCAGCAAGACAAGCAA  
                 r AGGCATCTGAGACTCCTCC | 831 |
| $\beta_4$  | f ATCAATGCCTCTGTTGTGT  
                 r CAAGCGTTTCCCTACTCTTG | 1230 |

2.6 Immunocytofluorescence

2.6.1 $\alpha_{1D}$ antisera

To determine the presence of $\alpha_{1D}$ protein in the stable HEK 293 $\alpha_{1D}$ cell line an $\alpha_{1D}$ polyclonal antiserum was raised in rabbit by Dr. N. Berrow (using standard techniques, described in Brickley et al., 1995). The antiserum was raised against a rat neuronal $\alpha_{1D}$
sequence at amino acids 1417-1434 (numbering according to rat brain sequence, accession no. M57682, Hui et al., 1991). The sequence to which the polyclonal antiserum was raised (KLCDPDPYNGEYTC) resides in the peptide loop between transmembrane segment S5 and S6 in domain IV and requires depolarisation of the membrane to be fully accessible from the outside of the cell by antiserum, unless cells are permeabilised (Wyatt et al., 1997). Though specific for rat brain αID, the degree of similarity of this sequence to the human αID sequence (there are two differences, KLCDPDPYNGEYTC) allows human αID protein to be detected. The sequence is also present in the rat skeletal muscle calcium channel αtS with one amino acid change (KLCDPDPYAPGEYTC), though, more importantly, no similar sequences are found in the alternative neuronal L-type calcium channel subunit, αtC.

2.6.2 Crude antibody purification (pre-absorption)

In order to purify the polyclonal anti-αID antiserum a crude purification protocol was performed. Essentially in the purification, or pre-absorption, process the antiserum is pre-incubated with blank HEK 293 cells allowing removal of antibodies interacting with other proteins, reducing the non-specific signal. The crude purification protocol was as follows: a 75cm² growing flask with a confluent monolayer of HEK 293 cells (approximately 7x10⁶ cells) was washed with 2 ml PBS and treated with 1 ml trypsin-EDTA to remove the cells. To this HEK 293 cell suspension 2 ml of DMEM was added and cells were pelleted by centrifugation (300 x g, 4 °C, 3 min). The supernatant was removed and the cell pellet was resuspended in 1 ml PBS and transferred to a 1.5 ml eppendorf tube. This was then repelleted (1000 x g; R.T., 21-25 °C; 3min), and the supernatant was discarded. This PBS cleaned cell pellet was then resuspended in 4% (w/v) paraformaldehyde in tris-buffered saline (TBS; consisting 20 mM Tris 7-9, 150 mM NaCl, pH 7.4), and fixed for 15 min at R.T. on a roller-mixer. The fixed cell suspension was once again pelleted (1000 x g, R.T., 3min), before being resuspended and permeabilised in 0.02% (v/v) Triton X-100 (Sigma) for 45 min on a roller mixer. After re-pelleting (1000 x g, R.T., 3 min) and removing the supernatant, the cells were resuspended in 1 ml of antiserum at a dilution of 1:500 in
antibody dilutant [consisting of 10 ml of 20% (v/v) goat serum (Sigma), 4% (w/v) bovine serum albumin (Sigma) and 0.1% (v/v) DL-lysine (Sigma), mixed with 10 ml of TBS]. This cell-antiserum mixture was then incubated for 2-4 h at R.T. on a roller mixer. The suspension was then centrifuged (13 000 x g, R.T., 4 min) and the supernatant (the crudely purified anti-α1D antiserum) was transferred to a fresh eppendorf tube and stored at -20 °C.

2.6.3 Immunocytochemistry protocol

The protocol used for determining the staining of α1D protein by the α1D antiserum was previously illustrated and described by Wyatt et al. (1995) in rat dorsal root ganglion neurons. In the experiments described here, HEK 293 (negative control) or HEK 293 α1D cells were removed from 25cm² growing flasks by trypsin-EDTA treatment and allowed to settle onto poly-L-lysine cover-slips for 30-45 min. Throughout this protocol all solution applications were at 200 μl (sufficient to entirely cover a 20 x 20 mm cover slip) and incubations were at R.T. (21-25 °C), unless otherwise stated. Adhered cells were then washed with TBS. To depolarise the cell membrane, the cells were incubated for 2 min at 37 °C in a modified Krebs-Henselit (K-H) solution (containing in mM: NaCl, 119; MgSO₄, 0.94; KH₂PO₄, 1.2; NaHCO₃, 25; glucose, 11.5; pH 7.4 and supplemented with 30 mM KCl). Immediately after this stage cells were fixed with 4% paraformaldehyde in TBS (supplemented with 30 mM KCl in membrane depolarisation experiments) for 30 min at R.T. In experiments where cell membranes were permeabilised, cells were washed for 3 x 5 min washes in 0.02% Triton X-100 in TBS. To prevent non-specific protein binding, the cells were incubated for 15 min with blocking serum [TBS containing 20% goat serum, 4% bovine serum albumin and 0.1% DL-lysine]. After removal of the blocking serum, the cells were incubated at 4 °C overnight with the crudely purified α1D subunit antiserum (1:500 dilution factor in antibody dilutant, see section 2.6.2 above). The cells were subsequently washed with blocking serum (4 x 5 min) and then incubated with a secondary antibody solution, consisting of goat anti-rabbit IgG conjugated to biotin (diluted 1:200 in antibody
dilutant, see above; Sigma) for 2 h at 4 °C. A further series of blocking serum washes (4 x 5 min) were followed by another incubation with a third conjugating agent (streptavidin fluorescein isothio-cyanate, FITC; 15 μg.ml⁻¹; pH 9.0; Molecular Probes, OR, USA) for 1 h. This fluorophore allows detection at a fluorescent excitation (absorbance) wavelength of 494 nm, with an emission wavelength of 514 nm. Unbound, excess fluorophore was washed with TBS (5 x 5 min), before mounting the cover-slip on a microscope slide with Vectorshield (Vector Laboratories, CA, USA). The cells were visualised using a confocal scanning laser microscope (Leica TCS SP, Leica Microsystems UK Ltd., Bucks., UK). To obtain comparable images uniform data acquisition parameters (aperture and gain) were maintained throughout.

2.7 Electrophysiology

2.7.1 Background to whole-cell patch clamp

Almost 25 years ago Neher and Sakmann (1976) showed that high resistance (50 MΩ) electrical seals could be gained by pushing a heat-polished glass pipette (containing a silver wire electrode surrounded by a designated electrolyte) against the membrane of frog skeletal muscle. The electrically isolated patch of membrane lying at the tip-aperture of the pipette may then be voltage-clamped (maintaining a set voltage) allowing accurate measurement of currents through this membrane patch. This provided a recording system whereby the signal to noise (electrical interference) ratio was sufficient to record single ion channels in the patch of membrane electrically isolated in the aperture of the pipette. This technique was dubbed cell-attached patch clamp. In subsequent years variations of this technique evolved whereby patches of membrane can be excised from the cell, allowing additional control of the solution on both sides of the membrane, by controlling the pipette solution and bathing solution. Inside-out and outside-out configurations of the patch clamping method allow specific localisation of the cytosolic facing membrane relative to the bathing solution (for variations in patch clamp configuration see Hamill et al., 1981).
Further refinements made to the patch clamp technique allowed electrical continuity of the pipette electrolyte solution (pipette solution, intracellular solution) with the cell interior (also allowing the whole cell to be voltage clamped). This was achieved by applying negative pressure (suction) and/or large voltage (1.5 V DC; 0.1-5 ms duration) to the pipette causing the membrane patch to rupture whilst maintaining the electrical and mechanical stability of the seal. In order to achieve such violent rupturing of the membrane whilst allowing maintenance of seal stability, improvements to the initial Neher and Sakmann patch clamp technique were made. These included ensuring the pipette tip surface was debris-free prior to seal formation (by applying positive pressure to the pipette) and on pressing the pipette onto the membrane a negative pressure was applied (aiding seal formation). The seals formed are unusually high resistance (10-100 GΩ). This gigaseal voltage clamping (whole-cell patch clamping) technique was initially described by Hamill et al., (1981). The whole-cell patch clamp technique was routinely used in the experiments described here.

Perforated patch-clamp recordings

The electrical continuity created by the pipette solution dialysing the cell contents has the advantage of allowing the cell contents to be controlled by the pipette solution. However, this dialysis can also create artefacts due to the ‘wash-out’ of cellular components. To overcome this problem pore-forming antibiotics can be added to the intracellular solution. These pore-forming agents create pores in the cell membrane that allow electrical continuity between the pipette solution and the cell membrane without loss of large cellular components (thus reducing the ‘wash-out’ effects associated with whole cell patch clamping). Nystatin (Horn & Marty, 1988) and amphotericin-B (Rae et al., 1991) are two such antibiotics commonly used for perforated patch clamp recordings, allowing small monovalent ions (such as Na⁺, K⁺ and Cl⁻) to pass through without movement of larger intracellular components (such as nucleotides and proteins, for example).
2.7.2 Patch clamp electronics: headstage and amplifier

A simplified circuit diagram showing the interactions between a whole patch-clamped cell and an operational amplifier is shown in Fig. 2.1. Essentially, the cell membrane, cell contents (dialysed with pipette solution), pipette solution and Ag-wire (coated with chloride; Advent Research Materials, Suffolk, UK) pipette electrode are contiguous with an operational amplifier. The Ag-wire pipette electrode continuously measures the membrane voltage and injects current simultaneously (unlike discontinuous, switch-clamp single electrode and two-electrode voltage clamp techniques). The perfusion bath in which the cell lies is earthed via a Ag/AgCl pellet (Clark Electromedical Instruments, Reading, UK) connected to an earthing socket located on the pipette holding headstage, completing the circuit. To prevent Ag⁺ ions leaching into the perfusion chamber the Ag/AgCl pellet was isolated from the chamber by encasing it in a salt bridge (see Fig. 2.1). The salt bridge was formed of a modified glass pasteur pipette filled with 3% (w/v) agar (Sigma) made in 1M KCl, allowing electrical continuity between the pellet and perfusion chamber whilst maintaining physical isolation. The operational amplifier acts as a current-voltage converter coupled to a high resistance feedback resistor (with a resistance, $R_f$, of 500 MΩ-50 GΩ, and a feedback capacitance, $C_f$, of approximately 0.1 pF, see Fig. 2.1). In the single electrode continuous voltage clamp technique used in this study, the voltage was measured at the tip of the silver wire electrode (other techniques, such as switch and two-electrode voltage clamp, measure at the glass electrode tip). The voltage measured at this point is formed of the membrane potential and an inherent voltage error (see below) that is inherent across the pipette. This voltage is continuously measured and updated via the injection of current, to maintain the voltage as designated by the signal input ($V_{ref}$). The feedback resistor across the operational amplifier controls the current injection to maintain the voltage clamp. An output from the differentiating amplifier allows online display and storage by a PC based acquisition system.
Throughout these experiments an Axopatch 1B or 200A amplifier (Axon Instruments, Foster City, CA, USA) and an Axopatch headstage (CV 201A, Axon) were coupled to a Digidata 1200 A/D converter interface (Axon). Data acquisition and voltage protocols were controlled via a Viglen P120 PC (Viglen, London, UK) using pCLAMP 6.02 software (Axon).
Capacitance compensation

Two forms of unwanted capacitance arise during recordings, and require compensation to prevent errors due to capacitance charging decreasing the speed and accuracy of voltage steps.

1). Pipette capacitance ($C_p$, see Fig. 2.1) is the combined capacitance of the pipette and the headstage (normally approximately 5pF). A slow and fast component of a capacitance arises due to slower charging arising from the pipette glass. Compensation of this $C_p$ will increase the rate of charging allowing the pipette potential (and hence voltage clamped membrane) to be stepped more rapidly and accurately; this compensation also removes capacitance ‘transients’ from the acquisition record. The commercial amplifiers used have ‘fast’ capacitance compensation circuitry, whereby a charging current can be injected (via a capacitor) to compensate this stray capacitance.

2). Another larger, stray capacitance arises from the capacitance of the cell membrane. Once again, the commercial amplifiers used have compensation circuitry, ‘slow’ capacitance compensation, which when set defines the whole cell membrane capacitance ($C_m$). The compensation provided is adequate for smaller cells (see below for $C_m$ values of the cells used in this study), though it can lead to membrane charging time errors in larger cells. $C_m$ is proportional to the amount of cell membrane, and therefore larger cells have greater $C_m$, and can be problematic in the rapid charging of such membranes.

An additional factor affecting the membrane charging time is the pipette access resistance ($R_{acc}$ in Fig. 2.1). The access resistance of the cell is defined as the sum of the pipette resistance and the residual resistance of the ruptured membrane patch. Since the access resistance forms the majority of the series resistance (see below), the series resistance (which can be compensated and measured on the amplifiers used) can be used as an accurate approximation of the access resistance. The relatively small size of the cells used in this study (GH4C1 D2, HEK 293/αiD and COS-7; $C_m$ routinely <20pF) and the good access achieved during whole cell recordings (routinely access resistance was <15 MΩ) resulted in
membrane voltage charging constant times of <300 μs. The fastest events studied here were at least several ms in duration and therefore the charging times were considered to be sufficiently small for the required time resolution. Since clonal cell lines were used in this study, acutely isolated (preventing the incorporation of additional resistance and capacitance due to electrical coupling to neighbouring cells) and spherical cells (avoiding space clamp errors) could easily be selected for recordings, therefore minimising these error sources. In cells where large access resistances (>15 MΩ) and \( C_m (>20 \text{ pF}) \) were encountered the membrane charging time was minimised by compensating the series resistance by >75%; where this was not possible the cells were not used.

**Series resistance (\( R_s \)) compensation**

The series resistance (\( R_s \)) comprises the resistance of the cell cytosol (\( R_{cell} \)) and the pipette access resistance (\( R_{acc} \)), shown in Fig. 2.1. The commercial amplifiers used have compensation circuitry which allow compensation of this \( R_s \). The \( R_s \) limits the speed of the voltage clamp and also results in voltage errors (see below). To compensate this loss of ideal electronic control the commercial amplifiers used correct the error by positive feedback ('prediction').

**Voltage-drop error**

The \( R_s \) creates an error in the measured membrane voltage when \( I \) flows across the membrane. This voltage error can be calculated according to Ohm's law, \( V=I.R_s \), and therefore is particularly problematic with large membrane \( I \). The currents studied routinely in this research were <200pA, and assuming the \( R_s \) was measured to be <15MΩ, then voltage errors would be calculated to be <3mV. This level of error was considered to be tolerable. However, some cells (especially transient transfections involving \( \alpha_{1B} \) and \( \alpha_{1E} \) VDCC subunits in HEK 293 and COS-7 cells) exhibited significantly larger currents. To reduce the subsequent voltage errors that these large currents create, series resistance compensation was set to >75%; if this was not possible the cell was not used for analysis.
Junction Potential

Further errors may arise due to a junction potential difference between the external bath solution and the intracellular patch pipette solution. The junction potential voltage offset was nulled using the amplifier, but on gaining whole-cell access the potential used to null the potential difference remains. Therefore the amplifier recordings must be corrected off-line for this junction potential offset. The differing concentrations and mobility constants of the ions present in each of the two solutions (intra- and extracellular) causes the junction potential at the pipette tip, and these parameters define the magnitude of this voltage offset. The junction potentials for various extracellular and intracellular solution (for full details of recording solutions see section 2.7.6) combinations are shown below in Table 2.1, and were measured according to the method described by Neher (1992). The method for measuring the junction potentials was as follows: the perfusion chamber and patch-pipette were both filled with intracellular solution and in I-clamp mode the voltage-meter reading was zeroed via the amplifier voltage offset potentiometer. The solution in the perfusion chamber was then removed and replaced by extracellular solution: the voltage meter on the amplifier defined the junction potential for this combination of solutions. The potentials throughout this study are uncorrected for the junction potentials.
Table 2.3 Measured junction potentials (in mV) for different combinations of extracellular and intracellular recording solutions.

<table>
<thead>
<tr>
<th>Intracellular solution</th>
<th>GH₄C₁D₂ Extracellular [10mM Ba²⁺]</th>
<th>Extracellular [10mM Ba²⁺]</th>
<th>Extracellular [20mM Ba²⁺]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Cell:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>✓ATP</td>
<td>-</td>
<td>-4.1</td>
<td>-2.2</td>
</tr>
<tr>
<td>✓MgCl₂</td>
<td>-</td>
<td>-2.7</td>
<td>-2.4</td>
</tr>
<tr>
<td>×Tris-GTP</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GH₄C₁D₂ Whole Cell:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High [Cl⁻]</td>
<td>-1.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GH₄C₁D₂ Whole Cell:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low [Cl⁻]</td>
<td>-1.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Perforated Patch</td>
<td>-3.1</td>
<td>-4.8</td>
<td>-5.4</td>
</tr>
</tbody>
</table>

2.7.3 Recording micro-electrodes (pipettes)

Recording micro-electrodes, or patch pipettes, were pulled from thick walled borosilicate glass capillary tubing with the following dimensions: 1.5 mm outer diameter, 1.0 mm bore diameter and with an internal 0.1 mm fibre (Plowden and Thompson, UK). The patch pipettes were initially pulled using a P-87 Flaming/Brown microelectrode puller (Sutter Instrument Company, Novato, CA, USA) followed by fire polishing of the pipette tip with a Narishige microforge (Tokyo, Japan). Pipettes fabricated in this way gave typical pipette resistances of 2-5MΩ (approximately 0.5-1μm tip diameter).
2.7.4 Data acquisition

As was mentioned in the previous section the data acquisition occurs via the patch clamp electronics composed of the patch pipette headstage, amplifier, A/D converter and subsequent storage on a PC. In addition to data storage the PC controls the amplifier voltage step outputs. These voltage step protocols were controlled by pCLAMP 6.02 software (Axon). The different voltage step protocols will be defined specifically as they occur in the Results sections (often as voltage pulse protocol schematics), though some general facets of the protocols can be discussed here. To prevent extraneous high frequency noise, currents were filtered by a 4 pole, low-pass Bessel filter (intrinsic to the amplifier) at rates of 1-2 kHz. Mindful of aliasing effects (according to the Nyquist Sampling Theorem acquisition rates should be >2 x filter rate, and ideally at least 5 x filter rate) the acquisition rates were set to 5-10 kHz (sampling every 100-200 µs). These sampling rates also allowed sufficient time resolution of the events being investigated. Voltage steps applied were made at intervals between 5-30 s (0.033-0.2 Hz).

In addition to the electronic/PC interfaces the experimental apparatus also included a microscope, patch pipette micro-manipulators and a gravity fed perfusion system.

Microscope

The necessity for easy access to the cell in the perfusion chamber dictates the use of an inverted microscope. In this study a Diaphot TMD inverted microscope (Nikon, Japan) with phase contrast (10X magnification lens, 10X eyepiece) was used, with a high pressure mercury lamp and designated fluorescent filter block to visualise GFP fluorescence (see section 2.4.1). To maintain mechanical stability of seals and to limit external mechanical noise on recordings the microscope was mounted on an anti-vibration air-table (TMC, Peabody, MA, USA). The electrical interference of recordings was limited by surrounding the experimental apparatus with a mesh Faraday cage.
Pipette micro-manipulators

A combination of coarse and fine hydraulic micro-manipulators (Narishige, Japan) were used to create precise movement of the pipette onto the cell membrane. This array of micro-manipulators allowed control to 0.2μm, sufficient for accurate pipette placement.

Gravity-fed perfusion system

The perfusion system was a home-made apparatus with a simple gravity-fed extracellular reservoir source, polyethylene supply line, perspex perfusion chamber (approximately 750μl volume) and an out-flow (polyethylene) connected to a peristaltic pump (Medcalf Bros. Ltd., UK) to remove waste perfusate. The flow rate was measured at 1-2 ml.min⁻¹, though for expensive compounds (e.g. α-CgTx GVIA) the rate could be slowed (0.5 ml.min⁻¹) or stopped by adjusting the height of the extracellular solution reservoir and/or clamping the in and out-flow lines. Efficient solution exchange of the entire perfusion chamber could be achieved in approximately 1 min.

2.7.5 Data analysis

Initial analysis of electrophysiological data was performed using Clampfit, part of the pCLAMP 6.02 software package (Axon Instruments). Additional analyses including calculation of means, sem, significance (paired or unpaired Student’s t-tests, where applicable) and curve fitting (according to equations shown below) were calculated using Lotus 1-2-3 (release 4, Lotus Development Corporation, Cambridge, MA, USA) and Microcal Origin 5.0 (Microcal Software Inc., MA, USA). Where mean values are presented they are shown as mean ± sem (with n depicting the number of cell recordings or experiments from which the mean was calculated). Statistical significance was defined as p<0.05 (*) and p<0.01 (**).

Equations

Curve fitting analysis was performed where appropriate using the fitting function of Microcal Origin (Microcal Software Inc.). The following three equations were used:
Equation 1

Where indicated current-voltage (IV) relation curves were fitted with a combined Boltzmann and linear fit function:

\[ I_{\text{density}} = G_{\text{max}}(V-V_{\text{rev}})/(1+\exp(-(V-V_{50(\text{act})})/k)) \]

where \( I_{\text{density}} \) is the current.pF\(^{-1}\) (of whole cell membrane capacitance), \( G_{\text{max}} \) is the maximum slope conductance.pF\(^{-1}\), \( V_{50(\text{act})} \) is voltage of the mid-point of activation, \( V_{\text{rev}} \) is the reversal potential and \( k \) is the slope factor for activation. This function provides a practical and simplified form of curve fitting approximating the \( V_{\text{rev}} \). The Goldman-Hodgkin-Katz voltage equation, based on the electrodiffusion theory (Goldman, 1943; Hodgkin & Katz, 1949), predicts current rectification at more depolarised potentials, modelling the rectification that is observed at these potentials. In this study the degree of rectification during voltage steps in the formulation of IV relationship curves is minimal and therefore a linear relationship for the reversal potentials provides a sufficiently accurate approximation of the \( V_{\text{rev}} \). Other factors that are important in the biophysical definition of the current (e.g. \( G_{\text{max}}, V_{50(\text{act})} \) and \( k \)) will be unaffected by this linear approximation of the reversing phase of the IV relationship.

Equation 2

Steady-state inactivation data were fitted with a Boltzmann function of the form:

\[ I/I_{\text{max}}=1/(1+\exp((V-V_{50(\text{inact})})/k)) \]

where \( I/I_{\text{max}} \) is the normalised peak current, \( V_{50(\text{inact})} \) is the voltage for the mid-point of inactivation, \( V \) is the conditioning voltage potential and \( k \) is the slope factor for inactivation.
Equation 3

Activation and inactivation of currents were investigated by fitting a single order exponential to the activating or inactivating phase of currents arising from an evoked step depolarisation (for an example of such a fit see Chapter 4, Figure 4.6A). The activating phase was defined as the area of the current from the start of the step depolarisation to the evoked peak current amplitude. The time-to-peak measurement was the duration of this activating phase (and therefore the time to 90% of peak, $\text{tt}_{90}$, was the duration of the activating phase required to reach 90% of the peak current amplitude). The current activation was fitted with the following function:

$$I = A_I \exp\left(\frac{-(t-t_0)}{\tau_{\text{activation}}}\right)$$

Where $A_I$ is the amplitude of the current at time $t_0$ in pA, $t$ is the time and $t_0$ is the time at the start of the fit in ms, $\tau_{\text{activation}}$ is the time constant for activation in ms. It should be noted that this fitting function actually represents an approximation of the current activation kinetics. Classical Hodgkin-Huxley (1952d) descriptions of current activation predict a sigmoidal activation curve. However the sigmoidal nature of activation is often partially masked by capacitance spikes (and to an extent gating currents) at the start of a depolarising voltage step. (This is particularly problematic in the recordings I have made due to the small size, generally <200pA, of currents such that even very careful capacitance compensation may not entirely remove capacitance spikes. In bigger currents the capacitance spike:current ratio is smaller, and therefore less masking of the sigmoidal activation will occur). Due to this masking, the single exponential fitting function used in this study provides an accurate approximation that reduces the artefactual characteristic associated with capacitance spikes. The rate of current inactivation was measured using a similar single exponential with the start of the fit occurring at the peak current amplitude (at amplitude $A_I$ and time $t_0$) and the end of the fit occurring at the end of the evoked current (prior to the tail current, at time $t$). The time constant of inactivation ($\tau_{\text{inactivation}}$) is exchanged in the equation for $\tau_{\text{activation}}$. 

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**On-line leak subtraction**

All the currents displayed and analysed are shown after leak-subtraction (unless stated). Currents were leak-subtracted on-line by pCLAMP 6.02 using the P/N protocol. Pulses were performed prior to voltage depolarisation steps with N values of 4 or 5. The P/N pulses were opposite in polarity to the pulse protocol (i.e. they were hyperpolarizing in nature) in order to prevent depolarisations that may have resulted in unwanted current activation prior to the test voltage depolarisation. The P/N protocol measures the currents resulting from N hyperpolarisations to 1/Nth of the overall voltage step. (For example, if a P/5 protocol were performed prior to a test step from -80 mV to +20 mV, currents would be measured from 5 hyperpolarizing steps to -100 mV). The resulting current measurements are summed and this resulting current is subtracted on-line. The P/N protocol assumes that leak currents are linear in nature, and thus if the leak at these hyperpolarizing potentials is non-linear it may introduce artefacts (see Chapter 3, Figure 3.4A, for an example of such non-linear leak currents which result in artefactual leak subtraction).

### 2.7.6 Recording solutions

The solutions were made using double distilled, de-ionised water (purified initially using an Elga Spectrum 2000 water purifier, Elga, UK; followed by another purification step using a MilliQ purification system, Millipore, France). All chemicals obtained were of high grade and obtained from Sigma, unless stated otherwise. Additional compounds and drugs were added from stock solutions (see Materials, section 2.1.1), where stated. All solutions were allowed to equilibrate to room temperature (R.T., 21-25 °C) before perfusion.

Isolation of currents through the calcium channels under investigation was possible using a combination of K⁺ and Na⁺ channel blockers [Cs⁺, tetra-ethyl ammonium (TEA), and tetrodotoxin (TTX)] in the intra- and extracellular solutions. TEA will act to block all the major K⁺ channel types [delayed rectifier, A-type, Ca²⁺-activated, inward rectifier and K(ATP)], whilst additional block was provided by the inclusion of Cs⁺ (reviewed in Hille, 1992). TTX, an alkaloid toxin found in pufferfish and porcupinefish, potently blocks most
sodium channels (reviewed in Catterall, 1980; Strichartz et al., 1987). The extracellular solutions routinely included Ba\(^{2+}\) at concentrations of 10 or 20mM, and occasionally replaced with the equivalent concentration of Ca\(^{2+}\) (allowing comparison of this physiological charge carrier). Relatively high concentrations of Ba\(^{2+}\) were required since current sizes were small (generally <200pA in 20mM Ba\(^{2+}\)) and by increasing the divalent ion concentration an increased single channel conductance can be obtained (Church & Stanley, 1996). Ba\(^{2+}\) was the divalent charge carrier of choice since Ba\(^{2+}\) shows greater permeability through calcium channels compared to Ca\(^{2+}\), producing larger currents (Fox et al., 1987a; Fox et al., 1987b). In addition using Ba\(^{2+}\) does not support the calcium-dependent inactivation associated with recording in Ca\(^{2+}\) (Chad & Eckert, 1986) and also aids the selection of Ba\(^{2+}\) currents by adding to the block of some K\(^{+}\) channel types [delayed and inward rectifiers, K(ATP), see Hille, 1992]. The inactivation effects of intracellular Ca\(^{2+}\) were further controlled by the inclusion in whole cell patch clamp solutions (irrelevant for perforated patch clamp since the pore size created would not allow access) of the Ca\(^{2+}\) chelator ethyleneglycol-bis \(\beta\)-aminoethylether (EGTA) (Fenwick et al., 1982; Byerly & Hagiwara, 1982). A further refinement of the whole-cell intracellular solution is the addition of ATP (Forscher & Oxford, 1985), which is thought to reduce irreversible current rundown, associated with intracellular solution dialysis ('wash-out', see section 2.7.1). GTP was also added to whole-cell intracellular solutions since GTP-free pipettes have been shown to abolish G-protein modulation effects (Lledo et al., 1990). In experiments where the non-hydrolysable GTP and GDP analogues were investigated, GTP was replaced by GTP-\(\gamma\)S (100-500\(\mu\)M; Sigma) or GDP-\(\beta\)S (2mM; Boehringer). The osmolarity of intra- and extracellular solutions was measured using a Wescor 5500 Vapour Pressure Osmometer (Logan, UT, USA), and was made to the specified osmolarity using sucrose. Intracellular solution was hypoosmotic in comparison to the extracellular solution: this aids gigaseal formation (Hamill et al., 1981). Intracellular solutions were stored as 400-500 \(\mu\)l aliquots
and stored at -20 °C, and used for a maximum of 2 recording days. Extracellular solution was stored at 4 °C in 1-2 l flasks and used within a maximum of 2 weeks.

**GH4C1 D2 recording solutions**

**Table 2.4** Composition of the intracellular solutions used to record I_{Ba} from GH4C1 D2 cells.

<table>
<thead>
<tr>
<th></th>
<th><strong>High [Cl] INTRACELLULAR</strong></th>
<th></th>
<th><strong>Low [Cl] INTRACELLULAR</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Constituent</strong></td>
<td><strong>Conc. (mM)</strong></td>
<td><strong>Conc. (mM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cesium chloride</td>
<td>140</td>
<td></td>
<td>Cesium aspartate</td>
<td>140</td>
</tr>
<tr>
<td>EGTA</td>
<td>3</td>
<td></td>
<td>EGTA</td>
<td>5</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>1</td>
<td></td>
<td>Magnesium chloride</td>
<td>2</td>
</tr>
<tr>
<td>HEPES</td>
<td>10</td>
<td></td>
<td>HEPES</td>
<td>20</td>
</tr>
<tr>
<td>TEA chloride</td>
<td>25</td>
<td></td>
<td>Calcium chloride</td>
<td>0.1</td>
</tr>
<tr>
<td>Magnesium ATP</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris-GTP</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**pH** adjusted with CsOH 7.3

**Osmolarity (mOsM)** adjusted with sucrose 300
Table 2.5 Composition of extracellular solution used to record $I_{Ba}$ from GH4C1 D2 cells.

<table>
<thead>
<tr>
<th>EXTRACELLULAR</th>
<th>Constituent</th>
<th>Conc. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Barium chloride</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Sodium chloride</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>Potassium chloride</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Tetrodotoxin (TTX)</td>
<td>$5 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

**pH**
- adjusted with NaOH: 7.3

**Osmolarity (mOsM)**
- adjusted with sucrose: 320

HEK293, HEK293 $\alpha_{1D}$ and COS-7 recording solutions

Table 2.6 Standard composition of the intracellular (left) and extracellular (right) recording solutions used to record $I_{Ba}$ (and $I_{Ca}$) from HEK293, HEK 293 $\alpha_{1D}$ and COS-7 cells.

<table>
<thead>
<tr>
<th>INTRACELLULAR</th>
<th>Constituent</th>
<th>Conc. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cesium aspartate</td>
<td>140</td>
</tr>
<tr>
<td></td>
<td>EGTA</td>
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</tr>
<tr>
<td></td>
<td>Magnesium chloride</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Calcium chloride</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>ATP</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>GTP</td>
<td>0.08</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EXTRACELLULAR</th>
<th>Constituent</th>
<th>Conc. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TEA bromide</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>Potassium chloride</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Magnesium chloride</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Sodium bicarbonate</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Barium (or Calcium) bromide</td>
<td>10/20</td>
</tr>
</tbody>
</table>

**pH**
- adjusted with CsOH: 7.2

**Osmolarity (mOsM)**
- adjusted with sucrose: 310

**pH**
- Sigma 7-9 adjusted: 7.4

**Osmolarity (mOsM)**
- adjusted with sucrose: 320
Perforated patch clamp recording solutions

The composition of the basic perforated patch clamp intracellular solution is given below in Table 2.7. Aliquotted nystatin or amphotericin stocks (see section 2.1.1) were added to a 500 μl aliquot of pipette solution to give a final concentration of 300 μg.ml⁻¹ and 240 μg.ml⁻¹ respectively. The nystatin pipette solution was used within 30 min of formation, whilst the amphotericin pipette solution could be used for up to 60 min; beyond these life-spans the effective pore-forming ability of the antibiotics would be reduced, limiting the electrical access attainable. Nystatin was used initially for recordings on GH₄C₁ D2 cells, though for subsequent recordings and on other cell lines amphotericin-B was used due to the improved electrical access and practical feasibility afforded by this antibiotic. The same extracellular solutions used for whole cell patch clamp recordings were used for the perforated patch clamp recordings.

Table 2.7 Composition of the perforated patch clamp intracellular recording solution.

<table>
<thead>
<tr>
<th>Perf. Patch INTRACELLULAR</th>
<th>Constituent</th>
<th>Conc. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cesium methan-</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>sulphonate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cesium chloride</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>Magnesium chloride</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>HEPES</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td><strong>pH</strong></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CsOH adjusted</td>
<td>7.3</td>
</tr>
<tr>
<td></td>
<td><strong>Osmolarity (mOsM)</strong></td>
<td>290</td>
</tr>
<tr>
<td></td>
<td>adjusted with sucrose</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 3

ELECTROPHYSIOLOGICAL INVESTIGATIONS OF THE G-PROTEIN MODULATION OF THE GH₄C₁ D2 CELL LINE
3.1 INTRODUCTION

The rat pituitary-derived GH₄C₁ D2 cell line was investigated using whole-cell and perforated patch clamp techniques. Initially GH₄C₁ D2 cells were used as an experimental model to determine the recording methodology, with particular reference to the most suitable recording solution for future experiments. For whole cell recordings, a high chloride intracellular solution was used at the start. However, unexpected leak-subtraction errors led to the use of a low chloride intracellular solution (based on a solution routinely used in Prof. Dolphin’s lab for recording COS-7 cell calcium channel currents). The first section of this chapter compares IV relationship curves for the high and low Cl⁻ intracellular solutions, and hence the logic for this choice. Another comparison was made in the GH₄C₁ D2 cells between the pore-forming antibiotics amphotericin-B and nystatin in order to determine the best and most feasible perforated patch method for future use.

Having determined some of the practicalities of the recording solutions, the currents of GH₄C₁ D2 cells were then characterised with some basic pharmacological tools. The predominantly L-type nature of the GH₄C₁ D2 calcium channel current was verified by the L-type specific DHP antagonist nifedipine. The non-specific calcium channel blocker Cd²⁺ was also applied. The G-protein modulation of the GH₄C₁ D2 calcium channel currents was investigated by application of the GPCR agonists: dopamine (DA) and quinpirole (Quin) for exogenous D2 receptor activation; somatostatin-14 (SS-14) for endogenous sst2 receptor activation; and carbachol (Carb) to activate endogenous muscarinic M4 receptors. Further G-protein modulation of the GH₄C₁ D2 calcium channel current was investigated by the intracellular dialysis of GTP-γS. G-protein inhibition was observed in the GH₄C₁ D2 calcium channel current once during perfusion of Quin (100 nM; 57 %), though this observation was never repeated with any of the various G-protein activating paradigms subsequently attempted.
3.2 RESULTS

3.2.1 Electrophysiological recording solutions

3.2.1.1 High versus low internal Cl⁻ concentration

Seabrook et al. (1994) performed experiments on the same clonal GH₄C₁ D₂ cell line and showed that 9 out of 21 cells displayed obvious modulation of the calcium channel current by activation of the human D₂₅ (huD₂₅) receptor pathway. During initial experiments, performed at Merck Sharp and Dohme (MSD) Research Laboratories, Harlow, a cell was shown to be responsive to quinpirole (100 nM; 57% inhibition, n = 1; see Figure 3.1). This cell was patched using the whole-cell configuration, with high Cl⁻, GTP and ATP present in the intracellular patch pipette solution. Subsequently, in my initial experiments attempting to repeat this observation, the same the high Cl⁻ with GTP/ATP supplementation intracellular solution conditions (see Methods, Table 2.4) were replicated. However, no calcium channel current modulation by huD₂₅ receptor activation was observed. A study by Lenz et al. (1997) suggested that high intracellular Cl⁻ could reduce the G-protein modulation of cationic conductances. As a result a low intracellular Cl⁻ solution (also defined in the Methods, Table 2.4), with or without nucleotide supplementation, was also used. Again no G-protein modulation of the GH₄C₁ D₂ calcium channel current was observed. It was noted, however, that IV relationships in these conditions reversed in a more linear nature than seen when using the high Cl⁻ intracellular solution: the high Cl⁻ solution appears to produce an unexpected rectification at positive test potentials. A series of IV relationships were performed using different intracellular conditions to investigate this observation further.
Figure 3.1 An example calcium channel current recording displaying quinpirole inhibition of peak $I_{Ba}$ of a cell recorded at MSD.

(A) A time course showing measured peak $I_{Ba}$ elicited by depolarisations to $-10$ mV from a holding potential of $-80$ mV (pulses occurring every 30s). The cell was patched in the whole cell configuration using the high Cl$^-$ intracellular solution supplemented with ATP and GTP; the charge carrier in the extracellular solution was 10 mM Ba$^{2+}$.

(B) The example traces were taken at numbered points along the time course. A trace recorded under control extracellular conditions (CTRL, point 1) and two showing the inhibition arising from quinpirole (Quin, 100nM, points 2 and 3) are shown.
Figures 3.2-3.6 represent a series of current-voltage (IV) relationship curves for the GH4C1 D2 cell line under different recording conditions, with measurements of mean peak barium current (I_{Ba}). Where applicable the IV relationship curves have been fitted by the modified Boltzmann equation (Equation 1).

Figure 3.2A shows the IV relationship for GH4C1 D2 cells recorded in the whole-cell configuration using the high Cl\(^-\) (167 mM) intracellular solution with GTP and ATP present, denoted by the filled squares. A cartoon depicting the IV relationship voltage step protocol is shown in Figure 3.2B. A comparison IV relationship is superimposed on the high Cl\(^-\) IV relationship: this second IV relationship was observed in GH4C1 D2 cells using intracellular solution with low Cl\(^-\) (4.1 mM) and lacking GTP or ATP (open triangles). I_{Ba} traces for each of the intracellular conditions are shown at test potentials of -25, -10 and +50 mV (examples for high Cl\(^-\) in Figure 3.2C; low Cl\(^-\) in Figure 3.2D). In conditions with high Cl\(^-\) and GTP/ATP present, very distinct, apparent inward rectification of the current occurs, with significant inward currents remaining even at high (+45 mV) depolarising potentials. In comparison low Cl\(^-\), and absence of ATP and GTP intracellularly, produces I_{Ba} with minimal rectification, and current reversal at depolarising potentials >+45 mV. This difference in rectification is mirrored in the example I_{Ba} traces: high Cl\(^-\) intracellular solution current traces at +50 mV are still comparatively large and inward. The high Cl\(^-\) traces also often show distinct kinetic properties, namely little or no inactivation, or continual activation over the 250 ms depolarising pulse at high, depolarised potentials (see the +50 mV example trace in Figure 3.2C). In the low Cl\(^-\), no ATP/GTP intracellular solution at the +50 mV test potential the I_{Ba} were usually at the point of reversal (as in the example, Figure 3.2D) or outward in nature, and did not show the continual activation kinetics displayed before in the presence of high intracellular Cl\(^-\) and nucleotides. This unexpected level of rectification seen with high Cl\(^-\) intracellular is further emphasised by the example traces shown in Figure 3.10A in which
Figure 3.2 Inward rectification observed with high Cl-, ATP and GTP intracellular but not with low Cl- (no ATP or GTP) intracellular solution.

(A) IV relationships for whole cell recordings with intracellular solutions comprising as follows: high Cl-, ATP/GTP (filled squares); and low Cl-, no ATP/GTP (open triangles). In each case modified Boltzmann fits (to the specified voltage ranges) have been made. (B) A schematic depicting the voltage step protocol: from a holding potential (V_hold) of -100 mV voltage steps from -90 mV to +50 mV (or -70 mV to +70 mV for the low Cl- intracellular solution), in 5 mV increments, were performed. Voltage steps were applied every 5 s for a duration of 250 ms. (C) Example traces for a recording from a cell using the high Cl- solution at potentials of -25 mV, -10 mV and +50 mV. Note the significant inward current at +50 mV. (D) Another set of example traces [at the same voltage steps for (C)] are shown, using the low Cl- intracellular solution.
a large depolarising pre-pulse to +120 mV still apparently produces inward current (see section 3.2.3.2). The parameters resulting from the curve fitting and peak $I_{Ba}$ densities are summarised in Table 3.1. The peak $I_{Ba}$ appears at a slightly more hyperpolarised potential for the high Cl\textsuperscript{-} compared to low Cl\textsuperscript{-} intracellular solution (-10 mV compared to -5 mV); similarly the $V_{50\text{act}}$ for high Cl\textsuperscript{-} is more hyperpolarised than for low Cl\textsuperscript{-} intracellular solution (-26.3 mV and -17.2 mV respectively).

To investigate the effects of holding current ($I_{hold}$) on the inward rectification observed when using high Cl\textsuperscript{-}/ATP/GTP intracellular solution two populations of cells were chosen (see Figure 3.3). One group of cells had $I_{hold}$ of between 1-10pA (filled squares); the second having larger $I_{hold}$ between 30-40pA (open squares). Both IV curves formed from this $I_{hold}$ selection process show very similar profiles of Boltzmann fit parameters and using an independent Student’s $t$-test were not significantly different (data not shown).

**Figure 3.3** The holding current ($I_{hold}$) does not affect the apparent inward rectification observed in high Cl\textsuperscript{-} intracellular conditions.

IV relationships for whole cell recordings with identical intracellular solution (high Cl\textsuperscript{-}, GTP and ATP). The IV curve with filled squares represents cells with low holding currents ($I_{hold}$); open squares represent cells recorded with high $I_{hold}$. The same voltage pulse protocol as described in Figure 3.1B was used to evoke the currents.
When the nucleotide supplements are removed from the high Cl⁻ intracellular solution, an IV curve with inward rectification results (open circles, Figure 3.4A). However, the degree of inward rectification is not as apparent as that displayed by high Cl⁻/GTP/ATP intracellular solution (compare to filled squares, Figure 3.2A). The IV curve with high Cl⁻, no GTP or ATP appears to be intermediate between those in Figure 3.2A. Another IV relationship displaying this intermediate degree of inward rectification was observed when the low Cl⁻ intracellular solution was supplemented with the non-hydrolysable analogue of GTP, GTP-γS (filled triangles, Figure 3.4A). Example current traces are displayed (Figure 3.4B) and were from a

**Figure 3.4** Intracellular conditions in which whole cell recordings produce IV relationships which were intermediate in their degree of inward rectification.

(A) IV relationships for low Cl⁻ with 100 μM GTP-γS (black triangles) and high Cl⁻, no ATP/GTP (open circles) display IV relationship kinetics that are intermediate to those shown in Figure 3.1A. For comparison, the extreme inward rectification observed in high Cl⁻ and 100 μM GTP-γS was also displayed (filled squares). The IV voltage pulse protocol depicted in Figure 3.1B was used. (B) Example traces from a recording using low Cl⁻ with GTP-γS intracellular solution are shown for voltage steps of -25 mV, -10 mV and +50 mV.
cell recorded with low Cl' and GTP-γS present. It can be noted in the example that a small, long lasting current at $V_t = +50$ mV was present which was unlike the persistently activating current observed at this potential with high Cl' and ATP/GTP intracellular solution (see Figure 3.2C). Addition of GTP-γS to the high Cl' intracellular solution creates evoked currents with an IV curve displaying large inward rectification (filled squares, Figure 3.4A), similar to that observed with high Cl', ATP/GTP intracellular solution (see Figure 3.2A, filled squares,). Thus, a picture emerged which suggested that high Cl' with ATP and GTP (or GTP-γS) present intracellularly were creating conditions in which apparent inward rectification was occurring at large depolarisations.

In previous studies in which IV relationships were shown in the closely related GH$_3$ cell line, no such inward rectification was observed (Barros et al., 1991; Piros et al., 1995; Zong et al., 1995b). Therefore this apparent inward rectification appeared to be either unique or aberrant. One identifiable possibility for the source of this apparent inward rectification is over-correction arising from the P/N leak subtraction. The set of IV relationship curves shown in Figure 3.5 investigated this possible error source. Figure 3.5A presents data recorded from GH$_3$C$_1$ D2 cells using the intracellular with high Cl'/ATP/GTP present. The IV relationship composed of open squares was leak-subtracted (P/N leak subtraction), and again clearly shows the unusual inward rectification at +50 and +60 mV. Strikingly, the IV relationship for recordings in the same high Cl'/ATP/GTP intracellular but without leak-subtraction (filled squares) displays more typical linear reversal between the IV relationship peak and through post reversal potential voltage steps. It is also apparent in Figure 3.5A that the ‘leak’ current observed at very hyperpolarised potentials (-110 mV to -130 mV) does not exhibit a linear relationship. A linear regression fit, denoted by a dotted line, for data points between -60mV and -100mV, emphasises the non-linear nature of ‘leak’ at these more hyperpolarised potentials. In direct comparison, when currents are measured using low Cl' intracellular solution (Figure 3.5B), with and without leak subtraction (open and closed triangles
respectively), it can be seen that in both paradigms the reversal of the IV relationship is more typically linear. Therefore, P/N subtraction does not introduce the apparent inward rectification with the low Cl⁻ intracellular solution. Further evidence is provided for the idea that the P/N subtraction is the source of the aberrant inward rectification shown previously, since in these low Cl⁻ intracellular conditions the ‘leak’ currents at large hyperpolarising potentials (-110mV to -130mV) are linear. Thus, the inward rectification that occurs with high Cl⁻ and ATP/GTP in the patch pipette appears to be due to over-subtraction resulting from non-linear ‘leak’ currents that are evoked at large (<-110mV) hyperpolarising potentials.

**Figure 3.5 IV relationships with and without P/N leak subtraction.**

The IV relationships displayed here were formed by using a voltage step protocol similar to that described in Figure 3.1B: the voltage step range was increased (-130 mV to +120 mV), and the voltage step increment was also increased (10 mV increments). Open data points have been leak subtracted with a P/N leak subtraction where N=5. A linear regression fit \(y=ax+b\) (dotted line) was applied to linear leak currents between -60 mV and -100 mV. (A) IV relationships using high Cl⁻, with ATP/GTP intracellular solution with leak subtraction (open squares) and without leak subtraction (closed squares). (B) The corresponding IV relationships for low Cl⁻ (with ATP supplementation) intracellular solution: leak subtracted records (open triangles) and non-leak subtracted records (closed triangles).
3.2.1.2 Amphotericin-B versus nystatin perforated patch

In order to investigate the feasibility of each of the pore forming antibiotics chosen for perforated patch clamp technique (amphotericin-B and nystatin), the basic biophysics of GH4C1 D2 calcium channel currents recorded under each of these conditions were determined by comparing the IV relationships for each. The IV relationship curves shown in Figure 3.6A are for perforated patch recordings using nystatin (crossed squares) and amphotericin-B (stars). The inset example traces shown (Figure 3.6B) are for an amphotericin-B perforated patch clamped cell. This voltage clamp configuration should prevent cytosolic ‘wash-out’ (including intracellular nucleotides) and minimise alterations to cellular internal milieu.

**Figure 3.6 IV relationships for perforated patch clamp recordings.**

The voltage step pulse protocol used was as described in Figure 3.1B, though for the nystatin recordings the voltage steps started at -60 mV and reached +90 mV by 10 mV incremental steps. (A) The mean data for nystatin perforated cells are depicted by crossed-squares, with a modified Boltzmann fit denoted by the dotted line; amphotericin-B perforated cell data (stars) with curve fit (solid line). (B) Current trace examples taken from an amphotericin-B perforated patch clamp recording at test step potentials of -25 mV, -10 mV and +50 mV.
although small monovalent ions such as Na⁺, K⁺ and Cl⁻ will equilibrate between pipette intracellular solution and cytosol (reviewed by Horn and Marty, 1988). The intracellular Cl⁻ concentration (31mM, see Methods, Table 2.7) in these experiments lies between the high (167mM) and low (4.1mM) Cl⁻ concentrations used in previous experiments. It can be seen that current reversal includes a degree of inward rectification at depolarised potentials in both perforated patch situations. Amphotericin-B perforated patches show peak $I_B$ at -10mV and $V_{50\text{act}}$ at -27.7 mV, whilst the nystatin appears to give peaks averaging at a more depolarised potential of 0mV and $V_{50\text{act}}$ at -14 mV (see Table 3.1). The apparent disparity in peak $I_B$ density (in pA.pF⁻¹) size may be due to differences in the apparent whole-cell capacitance ($C_m$) in each case (amphotericin-B 13.3 ± 2.6 pF; nystatin 24.2 ± 3.6 pF). When the actual $I_B$ size (in pA) is compared there is no significant difference in the recorded calcium channel current: for amphotericin-B recordings $I_B$ = -158 ± 37 pA, compared to nystatin recordings $I_B$ = -180 ± 27.

A summary of all the IV relationship peak $I_B$ densities and fitting parameters across the various intracellular conditions are displayed in Table 3.1. Most of the parameters were very similar. Some notable exceptions can be observed. The high Cl⁻ based intracellular solutions were all slightly more depolarised compared to the low Cl⁻ intracellular solutions. This was observed in the slightly more hyperpolarised peak $V_i$ potential (-10 mV with high Cl⁻, compared to -5mV with low Cl⁻) and $V_{50\text{act}}$ (between -21.2 and -26.3 mV for high Cl⁻, compared to -17.2 and -17.7 mV for low Cl⁻ intracellular solutions). Additionally, when comparing the two perforated patch antibiotics, amphotericin-B intracellular IV relationship parameters matched the whole cell parameters most closely.
Table 3.1  Summary of GH DC1 D2 cell IV relationship data parameters.

The peak $I_{Ba}$ and the fitting parameters arising from modified Boltzmann fits (Equation 1, see section 2.7.5) from mean calcium channel currents recorded in GH DC1 D2 cells are summarised in the table below. The far left column defines the basic intracellular solution components from IV relationships in Figures 3.2, 3.4 and 3.6. The first two columns show the test potential ($V_t$, in mV) at which peak current density was observed, followed by the mean±SEM peak current density (in pA/pF) measured at these $V_t$. The following four columns display the parameters that were calculated by fitting the data points with a modified Boltzmann equation (Equation 1). Using this fitting function the following parameters were estimated: maximal conductance ($G_{max}$); the test potential defining the mid-point of activation ($V_{50(act)}$); the reversal potential ($V_{rev}$); and the slope factor for activation ($k$). To provide practical estimates of $V_{rev}$, without the problems associated with inward rectification, modified Boltzmann fits were made to data points collected at depolarised potentials at which inward rectification was not apparent or minimal ($V_t = +25$ or $+30$ mV; see legends in Figures 3.2, 3.4 and 3.6). $V_{rev}$ was calculated from these fits by linear extrapolation.

<table>
<thead>
<tr>
<th>Intracellular recording conditions</th>
<th>$V_t$ for peak $I_{Ba}$ (mV)</th>
<th>Peak $I_{Ba}$ (pA/pF)</th>
<th>$G_{max}$ (pS/pF)</th>
<th>$V_{50(act)}$ (mV)</th>
<th>$V_{rev}$ (mV)</th>
<th>$k$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>high Cl^- + ATP/GTP n=45</td>
<td>-10</td>
<td>-13.3±1.1</td>
<td>274</td>
<td>-26.3</td>
<td>43.9</td>
<td>7.3</td>
</tr>
<tr>
<td>high Cl^- + GTP-γS n=3</td>
<td>-10</td>
<td>-15.6±3.2</td>
<td>276</td>
<td>-25</td>
<td>50.7</td>
<td>6.0</td>
</tr>
<tr>
<td>high Cl^- no ATP/GTP n=7</td>
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<td>-12.3±2.7</td>
<td>286</td>
<td>-21.2</td>
<td>41.4</td>
<td>7.7</td>
</tr>
<tr>
<td>low Cl^- no ATP/GTP n=7</td>
<td>-5</td>
<td>-11.4±3.7</td>
<td>302</td>
<td>-17.2</td>
<td>41.9</td>
<td>8.6</td>
</tr>
<tr>
<td>low Cl^- + GTP-γS n=7</td>
<td>-5</td>
<td>-13.9±2.4</td>
<td>307</td>
<td>-17.7</td>
<td>48.8</td>
<td>7.2</td>
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<tr>
<td>Amphotericin-B Perf. Patch n=8</td>
<td>-10</td>
<td>-12.9±2.3</td>
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<td>-27.7</td>
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<tr>
<td>Nystatin Perf. Patch n=10</td>
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<td>-8.0±1.0</td>
<td>171</td>
<td>-14</td>
<td>53.8</td>
<td>7.1</td>
</tr>
</tbody>
</table>
3.2.2 RT-PCR of GH₄C₁ D2 cell line VDCC subunit mRNA

The scanned gel shown in Figure 3.7 shows the migration by electrophoresis of amplification products of VDCC subunits from total RNA extracted from the clonal GH₄C₁ D2 cell line.

Each group of three lanes were loaded with amplification products for the α₁ VDCC subunits (A-E) in the upper half of the gel, and for β VDCC subunits (1-4) and actin in the lower half of the gel; the upper and lower halves of the gel have been delineated by a white dotted line. These three lanes have been loaded from left to right for each case in the following order: test (t, GH₄C₁ D2 cell cDNA used as amplification template), positive control (+, specific subunit cDNA, or rat DRG cDNA in the case of α₁b) and negative control (-, sterile, distilled water).

Actin, the overall positive control, has two amplification products loaded in the first two lanes (with a negative in the third). The first of these actin positive controls was amplified at an annealing temperature of 62°C, with the second being amplified at the more usual 65°C. A 100 base pair ladder marker preceded each of the three lanes for the specified VDCC subunit.

Each of the subunits shows a strong positive band, as expected, in the middle positive control lane (except for α₁b, and actin which has two positive products). α₁c, α₁d, β₁, β₂ and β₃ all show well-defined bands in the test lane (GH₄C₁ D2 cell extract, first lane) alongside the positive results. In addition α₁α and α₁e also display weak, but definite, bands in the test lane at the expected position (adjacent to positive bands). The β₄ amplification products do show a clear positive band, but no test band is visible. The α₁b shows no amplification products either from GH₄C₁ D2 cells or from the positive controls, though subsequent experiments in which the concentration of rat DRG cDNA (used as a positive control template in α₁b) was increased showed definite positive control bands [for both sets of α₁b primers, α₁b (1) and α₁b (2); see section 2.5.4] with no test α₁b detected (data not shown).
Figure 3.7 Gel electrophoresis of RT-PCR amplification products of VDCC subunits from RNA of a population of GH4C1 D2 cells.

The amplification products were electrophoresed on a 2% agarose gel and visualised using ethidium bromide staining and UV light. Upper half: amplification products for α1A-E, loaded (from left to right in each case) with test (t), positive (+) and negative (-) control; each set is separated by a 100 base pair (bp) ladder. Lower half: amplification products for β1-4 and actin. Once again, the order for each set is test, positive and negative (actin has two positive and one negative control) and each are separated by a 100 base pair ladder.
3.2.3 GH₄C₁ D₂ cell line pharmacology

3.2.3.1 GH₄C₁ D₂ calcium channel current sensitivity to GPCR pathways, DHPs and Cd²⁺

Following the GH₄C₁ D₂ cell calcium channel current that responded to Quin (100 nM) application by showing a rapid and striking inhibition (57 %, Figure 3.1), all subsequent recordings in which G-protein modulation was attempted failed to elicit any response. Examples of these experiments showing the lack of G-protein modulation are shown in Figures 3.8 – 3.10. Whole cell configuration calcium channel current recordings with high Cl⁻/ATP/GTP intracellular solution are shown in Figures 3.8A/D and 3.7B/E, whilst amphotericin-B perforated patch recordings are shown in Figures 3.8C/F. No inhibition was seen with DA (10µM) or Quin (1 µM) that might be expected to inhibit the I₇₅ via activation of the huD₂s receptor. SS-14 (100 nM), acting via the endogenous sst2 receptor, also exhibited no inhibition of the peak I₇₅. This pathway has been previously shown to inhibit calcium entry through S(-)-BayK8644 sensitive channels in GH₄C₁ cells (Liu et al., 1994). In addition to the lack of I₇₅ inhibition by these GPCR pathways, no change in kinetics was observed during GPCR agonist perfusion: trace 1 (control extracellular) and 2 (GPCR agonist in extracellular) overlap in each case. The 1,4-dihydropyridine, nifedipine (3µM), inhibits approximately 60% (trace 3) of I₇₅ in each of the three example cells. The current remaining after nifedipine washout was almost entirely removed by Cd²⁺ (100µM; approximately 90% inhibition; trace 4). The effect of Cd²⁺ could be rapidly washed off by control extracellular perfusion (trace 5).

The second endogenous GPCR pathway that was investigated to elicit the possible G-protein modulation of the L-type currents in the GH₄C₁ D₂ was the muscarinic M₄ receptor pathway. Once again, this M₄ receptor pathway, like the sst2 pathway, has been shown to have an inhibitory effect upon calcium entry through S(-)-BayK8644 sensitive calcium...
Figure 3.8  Time courses of drug action upon GH4C1 D2 calcium channel currents.

Filled circles denote peak $I_{\text{sh}}$ measured at test voltage step depolarisations ($V_t$) to -10 mV from a holding potential ($V_{\text{hold}}$) of -80 mV; test depolarisations were performed every 30 s with a sampling rate of 5 kHz. Black bars denote the duration of drug perfusion. The numbers 1-5 denote the data points used for example traces (on the right, D-F); in each case 1 was a control example; 2 with GPCR agonist; 3 with nifedipine; 4 with Cd$^{2+}$; and 5 final wash. (A) Whole cell recording using high Cl' (with ATP/GTP) showing the effects of dopamine (DA, 10 $\mu$M), nifedipine (NIF, 3 $\mu$M) and Cd$^{2+}$ (100 $\mu$M) perfusion. (B) As for (A) except with quinpirole (Quin, 1 $\mu$M) replacing DA perfusion. (C) Amphotericin-B perforated patch clamp recording showing the effects of SS-14 (100 nM), NIF (3 $\mu$M) and Cd$^{2+}$ (100 $\mu$M) perfusion. (D-F) Example traces relating to the numerically marked (1-5) data points in the time courses shown in Figures 3.7A-C. (D) Example current traces relating to the time course (A); the schematic above the example traces defines the voltage pulse protocol used in these time course experiments. (E) Current example traces for (B); and, (F) example traces for (C).
channels in GH₄C₁ cells (Liu et al., 1994). However, on perfusion of carbachol (Carb; 10 μM), a muscarinic receptor agonist, no modulation was observed during whole-cell patch clamp recordings using either the high Cl⁻ (n = 10) or low Cl⁻ intracellular solutions (n = 5). An example time course is shown in Figure 3.9: Carb perfusion has no effect on measured peak current or kinetics (see the inset example traces; trace 1 for control extracellular perfusion, trace 2 for Carb perfusion). Cd²⁺ (100 μM) perfusion, however, produces rapid and obvious inhibition (>90%) of the L-type component of the GH₄C₁ D2 cell line current (example trace 3). It can also be seen in this example trace (3) that after blocking the HVA component with Cd²⁺, a clear transient T-type current is unmasked. This is not wholly unexpected since GH₄C₁ cells are known to have T-type currents (first shown in GH₃ cells, from which the GH₄C₁ cell line was derived, Armstrong and Matteson, 1985). The clarity, however, of this example is unusual: when observed T-type currents were usually manifested as small transient currents at the start of long-lasting HVA currents (for examples see the current trace examples at the Vₕ = -25 mV in Figures 3.2C and 3.2D).

Figure 3.9 An example time course displaying the lack of effect during carbachol perfusion.

The recording was taken from a cell using the whole cell patch clamp technique using a low Cl⁻ based intracellular solution (with ATP), with a voltage step protocol as defined in Figure 3.7D. The perfusion of carbachol (Carb, 10 μM) and Cd²⁺ (100 μM) are designated by the black bars. The inset shows example traces of currents during control extracellular solution (1), Carb (2) and Cd²⁺ (3) perfusion.
To summarise, a series of recordings were made from cells under different recording conditions including whole-cell and amphotericin-B perforated patch clamp configurations, various holding potentials ($V_{\text{hold}}$: -60, -80 and -100 mV) and intracellular solutions (high and low Cl$^-$ intracellular solutions) for whole cell and perforated patch clamped experiments. A summary of drug effects at different concentrations in the different recording protocols are summarised in Table 3.2. Two GH$\text{C}_{1}$D2 cells were investigated for Quin (100 nM) inhibition of calcium channel currents at MSD (Harlow): a cell patched with high Cl$^-$/ATP/GTP intracellular in the whole cell patch clamp configuration displayed obvious inhibition in response (57%); the other cell which was recorded using the nystatin perforated patch clamp configuration displayed no response. All the other experiments were performed at UCL, are summarised as follows: no inhibition was observed with quinpirole (Quin; 100 nM - 1 μM; $n = 45$) and dopamine (DA; 10 μM; $n = 5$). Nifedipine (NIF; 3 and 10 μM; $n = 19$) inhibited approximately 60% of $I_{\text{Ba}}$ with 10 μM showing slightly more inhibition (approximately 70%; $n = 3$) at the lower $V_{\text{hold}}$ of -60 mV. The non-selective HVA VDCC blocker Cd$^{2+}$ inhibited approximately 90% of $I_{\text{Ba}}$ ($n = 28$). SS-14 (100 nM), an agonist of the endogenous sst2 receptor, showed no inhibition of $I_{\text{Ba}}$ ($n = 5$). This lack of response was also observed for another endogenous GPCR, the M4 receptor, when Carb (10 μM) was perfused ($n = 10$ using high Cl$^-$ intracellular; $n = 5$ using low Cl$^-$ intracellular solution).
Table 3.2  Summary of the \( \text{GH}_4 \text{C}_1 \text{D2} \) cell line current pharmacology.

The figures, where applicable, denote the mean± sem (figures in brackets denote \( n \) numbers for individual cell recordings) inhibitions for different recording extracellular solutions under different recording protocols (whole cell and perforated patch clamp techniques; varying holding potentials, \( V_{\text{hold}} \)) and conditions (high and low Cl\(^-\) intracellular solutions). Recordings were made from between 1-9 separate experimental preparations (each preparation a cover-slip coated with \( \text{GH}_4 \text{C}_1 \text{D2} \) cells from a passage different from the next). Figures highlighted by * indicate recordings made at MSD (Harlow); \( \text{NYSTAT} \) indicates that the nystatin perforated patch clamp technique was used.

<table>
<thead>
<tr>
<th>% Inhibition±sem (n); Recording protocol/conditions</th>
<th>Whole cell High Cl(^+)/ ATP/GTP ( V_{\text{hold}}=-80\text{mV} )</th>
<th>Whole cell High Cl(^+)/ ATP/GTP ( V_{\text{hold}}=-60\text{mV} )</th>
<th>Perf. patch Amphot.-B ( V_{\text{hold}}=-80\text{mV} )</th>
<th>Whole cell Low Cl(^-) ( V_{\text{hold}}=-100\text{mV} )</th>
<th>Whole cell Low Cl(^-) ( V_{\text{hold}}=-80\text{mV} )</th>
<th>Whole cell Low Cl(^-)/ 500( \mu )MGTP-( \gamma )S ( V_{\text{hold}}=-100\text{mV} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quin. (100nM)</td>
<td>0 (15)</td>
<td>57 (1*)</td>
<td>0 (1*NYSTAT)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quin. (200nM)</td>
<td>0 (8)</td>
<td>-</td>
<td>0 (5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quin. (300nM)</td>
<td>0 (2)</td>
<td>-</td>
<td>-</td>
<td>0 (3)</td>
<td>-</td>
<td>0 (7)</td>
</tr>
<tr>
<td>Quin. (1</td>
<td>( \mu )M)</td>
<td>0 (5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DA (10</td>
<td>( \mu )M)</td>
<td>0 (5)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NIF (3</td>
<td>( \mu )M)</td>
<td>56.2±9.9 (7)</td>
<td>59.5 (2)</td>
<td>64.1±2 (5)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NIF (10</td>
<td>( \mu )M)</td>
<td>59.9 (2)</td>
<td>71±3.1 (3)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cd(^{2+}) (100</td>
<td>( \mu )M)</td>
<td>87.5±1.4 (12)</td>
<td>90 (2)</td>
<td>89.7±8.2 (6)</td>
<td>-</td>
<td>97.7±1 (5)</td>
</tr>
<tr>
<td>Carb. (10</td>
<td>( \mu )M)</td>
<td>0 (10)</td>
<td>-</td>
<td>-</td>
<td>0 (5)</td>
<td>-</td>
</tr>
<tr>
<td>SS-14 (200nM)</td>
<td>0 (4)</td>
<td>-</td>
<td>0 (1)</td>
<td>-</td>
<td>0 (4)</td>
<td>-</td>
</tr>
</tbody>
</table>
3.2.4 Direct G-protein activation by intracellular GTP-γS does not modulate the GH4C1 D2 calcium channel current

In the previous section results were shown for experiments attempting to elicit G-protein modulation of the GH4C1 D2 calcium channel current by exogenous (huD2s) and endogenous (sst2 and M4) GPCRs. In performing such experiments a number of assumptions are made about the GH4C1 D2 cell line. In order to transduce the agonist signal the following critical features are required: the GPCR must be functionally expressed; the specific G-protein subunit isoforms that link the activated GPCR and VDCC must also be present; and, accessory protein(s) are often needed for efficient GPCR/G-protein/VDCC coupling (e.g. AKAPs). Several studies have shown that these features are present in the GH4C1 cell line (see the Introduction, section 1.6), and that they are all functionally coupled since G-protein modulation of the L-type current component has been observed (Liu et al., 1994; Seabrook et al., 1994; reviewed by Albert and Morris, 1994). A more direct method of G-protein activation can be achieved by utilising the non-hydrolysable GTP analogue, GTP-γS, in the intracellular pipette solution. Using this direct activation protocol the entire population of G-proteins in the cell are effectively activated, overcoming any possible problems of inefficient coupling of the GPCR to the GPCR agonist or VDCC. This activation assumes constitutive exchange of GDP for GTP-γS: without GPCR activation this exchange may be slow, though due to the non-reversible fashion in which GTP-γS binds to the Gα subunit, exchange favours the accumulation Gα–GTP-γS and free Gpγ moieties. The accumulation of Gpγ and resulting VDCC inhibition can be determined by the temporary removal of Gpγ subunits from the VDCC complex by large depolarising (> +90 mV) pre-pulses prior to the test voltage step (Bean, 1989). The removal of activated Gpγ causes the current to temporarily facilitate: this pre-pulse facilitation can be measured by calculating the ratio between the noPP current (the current evoked without a pre-pulse) and the +PP current (the current evoked following a large
depolarising pre-pulse). Thus the if the resulting +PP/noPP ratio >1, then the current displays pre-pulse facilitation; if +PP/noPP <1 then pre-pulse inhibition is defined; if +PP/noPP=1 then the pre-pulse has had no effect. Due to the small size of calcium channel currents recorded (<300 pA) in the GH4C1 D2 cells a variation of ±10% between noPP and +PP currents was considered to be within the limits for no apparent effect.

Figure 3.10 shows calcium channel current recordings from cells with an intracellular pipette solution supplemented by GTP-γS, which would be expected to elicit G-protein modulation of the current, if the current was susceptible to G-protein modulation. Figure 3.10A displays example current traces from a cell recorded with high Cl⁻ intracellular solution supplemented with 500 µM GTP-γS. The schematic above the current traces shows the voltage pulse protocol used to evoke the currents: the noPP current was evoked by depolarising from a holding potential (V_hold) of -80 mV to a test step potential (V_t) -20 mV in the example given. The +PP current was evoked 15 s later, with the standard test voltage potential step (-20 mV) preceded by a large depolarising step (+120 mV); the gap between the test step pulse and the large depolarising pre-pulse varied between 7.5-22 ms (20 ms in this example). To allow comparison between currents evoked in this cell line (and other cell lines) the standard voltage step at which pre-pulse facilitation measurements were made was 10 mV more hyperpolarised than the observed peak calcium channel current. In GH4C1 D2 cells peak calcium channel current was commonly measured at -10 mV, hence pre-pulse facilitation determination was generally measured at -20 mV. This voltage allows sufficient current activation to provide accurately recordable currents, whilst minimising the current inactivation that can occur at higher voltages (and hence minimise the possibility of masking G-protein pre-pulse facilitation). It can be seen that at the -20 mV test step potential no facilitation is observed (+PP and noPP currents are equal, therefore +PP/noPP=1). During the depolarising pre-pulse to +120 mV (in the voltage step used to elicit the +PP current) it can be seen that an unexpected inward current is apparent (*). As alluded to in section 3.1.1 (and discussed
Figure 3.10  Example $I_{Ba}$ traces showing the effects of large depolarising pre-pulses on calcium channel currents recorded from GH4C1 D2 cells patched with GTP-γS in the intracellular recording solution.

Cartoons above each set of traces depict the voltage pulse protocols which elicited the $I_{Ba}$ traces shown. (A) Whole cell configuration with 500 μM GTP-γS added the high Cl⁻, GTP/ATP intracellular solution. Note the unexpected inward current during the depolarising pre-pulse (highlighted by *, +PP current). (B) Whole cell configuration with 100 μM GTP-γS added to the low Cl⁻ intracellular solution (no GTP/ATP).

Further in section 3.3.1.1) this appeared to be due to over-subtraction of current records by the P/N leak subtraction.

To ensure that pre-pulse facilitation effects were not missed due to the pre-pulse protocol used or due to the P/N leak subtraction errors associated with high Cl⁻ intracellular solution, a different pre-pulse facilitation voltage step protocol was used, and recordings were repeated using a low Cl⁻ based intracellular solution. An example of such an experiment is shown in Figure 3.10B. The schematic above the example traces shows the voltage pulse protocol used. This voltage pulse protocols allows a range of voltages to be studied in an
attempt to observe pre-pulse facilitation. However in this typical family of traces no pre-pulse facilitation occurs, and early voltage steps (at -40 mV and -30 mV) display pre-pulse inhibition.

Variations in test step potential (-40 to +20 mV), depolarising pre-pulse potential amplitude (+90 to +120 mV) and duration (75 to 112.5 ms), and the time delay between pre-pulse and test-pulse (7.5 to 22 ms) were all made to determine whether facilitation could be observed. These parameters can all have effects on the degree of facilitation observed (Feldmeyer et al., 1992). However, no specific protocol was found which allowed detection of any facilitation. Table 3.3 lists the series of intracellular and extracellular conditions under which pre-pulse protocols were attempted. Whether G-protein activating compounds, such as perfusion of GPCR agonists (Quin, SS-14 or Carb) or intracellular supplementation with GTP-γS were included or not, no pre-pulse facilitation was detected. This table also lists the observed lack of inhibition of I_{Ba} for GPCR agonist application (with and without intracellular GTP-γS). Once again (as was observed in the summary of pharmacology in Table 3.2, excepting the single cell current displaying Quin inhibition), Table 3.3 shows that across all the G-protein activating conditions no G-protein modulation was observed in the calcium channel current found in GH4C1 D2 cells.

Further evidence for the lack of modulation by direct GTP-γS G-protein activation is displayed in the IV relationships when GTP-γS is present (see Figure 3.4A). If channels are G-protein modulated, typical characteristics of G-protein modulation of the IV relationship include: a decrease in peak current (density) and a depolarising shift in the IV relationship (Bean, 1989). By comparing these parameters for IV relationships with and without GTP-γS inclusion (see Table 3.1), it is apparent that no such G-protein modulation is occurring with the GH4C1 D2 cell line calcium channel current. High Cl^{-} intracellular solution with no ATP/GTP gave a mean IV relationship with a \( V_{50(\text{act})} = -21.2 \) mV and peak current density (at a voltage step of -10 mV) of 12.3 ± 2.7 pA.pF^{-1} (n = 7); a high Cl^{-} intracellular solution with
ATP/GTP and supplemented by 100 μM GTP-γS was not significantly different with a \( V_{50(act)} = -25 \text{mV} \) and peak current density (at -10 mV) of 15.6 ± 3.2 pA.pF\(^{-1}\) \((n = 3)\). In low Cl\(^-\) intracellular solutions no G-protein modulation was also apparent. A low Cl\(^-\) intracellular solution with no ATP/GTP gave \( V_{50(act)} = -17.2 \text{mV} \) and peak \( I_{Ba} \) density = 11.4 ± 3.7 pA.pF\(^{-1}\) (at -5 mV; \( n = 7 \)); whilst with 100 μM GTP-γS supplementation gave \( V_{50(act)} = -17.7 \text{mV} \) and peak \( I_{Ba} \) density = 13.9 ± 2.4 pA.pF\(^{-1}\) (at -5 mV; \( n = 7 \)).

### Table 3.3 Summary of the effects of G-protein inhibition by GPCR activation and of the large depolarising pre-pulse (+PP/noPP Effect) on the GH\(_4\)C\(_1\) D2 cell line calcium channel current.

A range of intracellular and extracellular recording solutions is summarised \((n\) numbers in brackets). Inhibitions for 10 mM Ba\(^{2+}\) perfusion are not applicable (N/A) since this is the control extracellular solution, though +PP/noPP effects are noted since the large depolarising pre-pulse can remove tonic G-protein modulation.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Extracellular</th>
<th>( I_{Ba} ) Inhibition ((n))</th>
<th>+PP/noPP Effect ((n))</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intracellular</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No GTP-γS; high Cl(^-)</td>
<td>10mM Ba(^{2+})</td>
<td>N/A</td>
<td>NONE (5)</td>
</tr>
<tr>
<td></td>
<td>SS-14 (200nM)</td>
<td>0 (4)</td>
<td>NONE (2)</td>
</tr>
<tr>
<td></td>
<td>Carb (10μM)</td>
<td>0 (10)</td>
<td>NONE (2)</td>
</tr>
<tr>
<td>No GTP-γS; low Cl(^-)</td>
<td>SS-14</td>
<td>0 (4)</td>
<td>NONE (4)</td>
</tr>
<tr>
<td></td>
<td>Carb (10μM)</td>
<td>0 (5)</td>
<td>NONE (4)</td>
</tr>
<tr>
<td></td>
<td>Quin (300nM; ( V_{hold} = -100 \text{mV} ))</td>
<td>0 (3)</td>
<td>NONE (3)</td>
</tr>
<tr>
<td><strong>100μM GTP-γS; high Cl(^-)</strong></td>
<td>10mM Ba(^{2+})</td>
<td>N/A</td>
<td>NONE (13)</td>
</tr>
<tr>
<td></td>
<td>Quin (100nM)</td>
<td>0 (2)</td>
<td>NONE (2)</td>
</tr>
<tr>
<td></td>
<td>Quin (200nM)</td>
<td>0 (2)</td>
<td>NONE (2)</td>
</tr>
<tr>
<td><strong>100μM GTP-γS; low Cl(^-)</strong></td>
<td>Quin (300nM)</td>
<td>0 (7)</td>
<td>NONE (7)</td>
</tr>
<tr>
<td><strong>500μM GTP-γS; high Cl(^-)</strong></td>
<td>10mM Ba(^{2+}) (( V_{hold} = -60 \text{mV} ))</td>
<td>N/A</td>
<td>NONE (6)</td>
</tr>
<tr>
<td></td>
<td>10mM Ba(^{2+})</td>
<td>N/A</td>
<td>NONE (16)</td>
</tr>
</tbody>
</table>
3.3 DISCUSSION

3.3.1 GH4C1 D2 recording solutions

3.3.1.1 IV relationships - low Cl\textsuperscript{–} intracellular solution supplemented with ATP and GTP was chosen for the intracellular recording solution

It is apparent throughout the IV relationships that low Cl\textsuperscript{–} and absence of nucleotides (ATP, GTP or GTP-\textgamma S) intracellularly results in an IV curve with minimal inward rectification and linear reversal (for an example see Figure 3.2A, open triangles). Intracellular conditions where nucleotides are present alongside high Cl\textsuperscript{–}, however, produce large, significant inward rectification at high depolarising potentials (+40 mV and higher; see filled squares, Figure 3.2A; and filled squares, Figure 3.4A). When either nucleotides are present (filled triangles, Figure 3.4A) or high Cl\textsuperscript{–} (open circles, Figure 3.4A) independent of each other then the apparent inward rectification appears intermediate between the two extremes (linear reversal observed with low Cl\textsuperscript{–} and the significant apparent inward rectification observed with intracellular containing high Cl\textsuperscript{–} and nucleotides). Further to this observation, a similar intermediate degree of inward rectification was seen in the perforated patch experiments (Figure 3.6), where intracellular Cl\textsuperscript{–} concentration was intermediate between the low and high Cl\textsuperscript{–} intracellular solutions (31 mM compared to 4.1 and 167 mM respectively). The inward rectification does not appear to be affected by I\textsubscript{hold} since the two cell populations in Figure 3.3 are under identical conditions differing only in I\textsubscript{hold} (cells represented by open squares have mean I\textsubscript{hold} approximately 5 times the size of those represented by filled squares) and have almost identical IV relationship curves.
‘Inward rectification’ in high Cl⁻ intracellular solution arises due to erroneous P/N leak subtraction

These data suggest that the apparent inward, rectifying current observed at large depolarisations was affected by the intracellular concentration of nucleotides and Cl⁻. This result was unexpected and aberrant, and therefore suggested there may be problem with the recording protocols or circuitry. A possibility for this apparent aberration was erroneous leak subtraction. An assumption that is implicit in the calculation of leak-subtraction by the P/N protocol is that ‘leak’ measured at the hyperpolarised potentials is linear in nature. However, when the currents were measured over a large range of hyperpolarising and depolarising steps (-130 mV to +120 mV) with and without leak subtraction it can be seen that linear ‘leak’ currents were not present at large hyperpolarising potentials (hyperpolarised to -110 mV) in the non-leak subtracted recordings made in high Cl⁻ with ATP/GTP intracellular solution (see Figure 3.5A, filled squares). This non-linear leak current at these hyperpolarised potentials was emphasised by the linear regression fit to linear leak currents at potentials between -60 and -100 mV: data points between -110 and -130 mV do not lie on this linear leak current fit in high Cl⁻ intracellular solution recordings. In contrast, using a low Cl⁻ intracellular solution with only ATP present, linear ‘leak’ currents are apparent at these large hyperpolarised voltage steps (filled triangles, Figure 3.5B). Thus, in the high Cl⁻ with ATP/GTP intracellular solution the non-linear ‘leak’ currents will cause an error when the currents are leak subtracted: over subtraction of the current will cause the currents evoked at >+30 mV to appear inwardly rectifying. These voltages were affected since the total voltage step of >130 mV (that is from -100 mV to >+30 mV) provides sufficient voltage, such that when leak subtraction pre-pulses are calculated and subtracted (for example, a P/5 subtraction, 26 mV hyperpolarised to the holding potential of -100 mV) they were taking place within the non-linear ‘leak’ current voltage range (hyperpolarised to -126 mV according to the example calculation). In low Cl⁻ intracellular solution such errors do not occur since the ‘leak’ current at these hyperpolarised potentials is linear in nature, therefore over-subtraction does not arise.
Possible current sources producing the non-linear leak current and subsequent leak subtraction error

In the previous section it was suggested that the source of the inwardly rectifying current observed in high Cl⁻ and ATP/GTP intracellular solution was due to non-linear leak currents at hyperpolarised (<-110 mV) potentials. These non-linear leak currents are likely to be due to the activation of an ion channel allowing a contaminating current to compromise the linearity of the leak current at these hyperpolarised potentials. Though an in depth biophysical or pharmacological study of this contaminating current was not attempted, being beyond the scope of the present study, some discussion on possible candidates is possible.

An initial candidate for the contaminating current that is activated at the large hyperpolarising potentials at which the non-linear leak current occurs is the Hyperpolarisation activated, Cyclic Nucleotide gated (HCN) family of channels. These channels are permeable to Na⁺ and K⁺ ions (found at concentrations of 155 mM and 5 mM respectively in the GH₄C₁ D2 extracellular solution, see Methods, Table 2.5) and create inward current via these charge-carrying ions at potentials hyperpolarised to -50 to -70 mV. However, the pharmacology that is evident in the contaminating current, namely a sensitivity to intracellular Cl⁻ and ATP/GTP (HCN channels are sensitive to GTP, though it reduces the channel current, and hence opposite to the effect needed for the explanation here), is not displayed by this channel family (for a review of the HCN family pharmacology see Pape, 1996). Therefore, the HCN channels, though obvious candidates, are not the likely source of the contaminating current.

The most likely source for the apparent “inward” rectifying current at large hyperpolarised voltage steps is an efflux of Cl⁻, possibly modulated by ATP and/or GTP: this may explain the effects displayed in the presence of high intracellular Cl⁻ and nucleotides. One possibility that exhibits many of the necessary traits is the hyperpolarisation activated Cl⁻ conductance (G_{Cl(V)}, Staley, 1994). However, Staley (1994) showed that removal of ATP or GTP from the intracellular recording solution had no effect on this Cl⁻ conductance, and
therefore does not exhibit nucleotide sensitivity that was observed in the contaminating current in this study. Two further Cl⁻ channels that are activated by hyperpolarising voltage steps are the cloned chloride channel CIC-2 and the protein phospholemman (for reviews see, Pusch and Jentsch, 1994; Hume et al., 2000). Phospholemman induced chloride currents are blocked by extracellular Ba²⁺ (Moorman et al., 1992), suggesting therefore that CIC-2 may be the candidate for the source of the contaminating current. The CIC-2 channel activates at very large hyperpolarising potentials (< -100 mV); it is ubiquitously expressed in many tissues and clonal cell lines; and, CIC-2-like currents in intralobular duct cells of mouse mandibular glands are activated by increasing concentrations of intracellular Cl⁻ (Dinudom et al., 1993).

ATP may also activate the CIC-2 channel by the PKA pathway: PKA stimulation of a gastric CIC-2 homologue (CIC-2G) has been reported (Sherry et al., 1997), though expression of CIC-2 in Xenopus oocytes produced currents that were insensitive to PKA (Furukawa et al., 1998c). However despite the uncertainty regarding CIC-2 sensitivity to ATP, all the other CIC-2 characteristics match the contaminating current closely. Thus, the CIC-2 channel appears to be the most convincing candidate for the inwardly rectifying current that prevents linear leak current at potentials < -110 mV, and the subsequent erroneous P/N leak subtraction observed in GH₄C₁ D2 cell line IᵥBa.

Low Cl⁻ intracellular solution was used to limit erroneous leak subtraction and provide accurate recordings

In Figure 3.5A it can be seen that deviation from linear leak currents occurs at potentials hyperpolarised to -110 mV in high Cl⁻ and ATP/GTP intracellular solution. Thus erroneous over-leak subtraction will occur during voltage step protocols where these large hyperpolarised potentials are surpassed (e.g. during leak subtraction of large depolarising prepulse records, see below).

To improve the accuracy of the recordings and prevent this over-subtraction, the concentration of Cl⁻ in the intracellular solution was reduced. Substantial experimentation was
performed using the high Cl⁻ based intracellular solution since the initial GH₄C₁ D₂ cell displaying G-protein current inhibition had been recorded in these conditions (Figure 3.1). However, as increasing data accumulated using these high Cl⁻ recording conditions it became apparent that further G-protein modulation of currents was unlikely to be observed. Thus, in addition to preventing the leak subtraction errors, reduction of intracellular Cl⁻ was attempted as an alternative recording environment. This, it was hoped, may aid the observation of possible G-protein modulation effects since elevated levels of intracellular Cl⁻ had previously been shown to reduce the effects of G-protein modulation (Lenz et al., 1997). Further support for this change of intracellular solution is apparent in the observation that a low Cl⁻ intracellular solution is also routinely used for whole cell patch clamp experimentation investigating G-protein modulation of VDCC in mammalian expression systems (COS-7 and HEK 293 cells), with no detrimental effects observed.

Although nucleotides were also shown to have some effects upon the erroneous inward rectification, nucleotide inclusion was maintained. There are a number of reasons for the nucleotide inclusion:

(i) Pipettes filled with an intracellular solution lacking GTP have been shown to prevent G-protein modulation (Lledo et al., 1990).

(ii) The presence of GTP and ATP in the intracellular pipette solution was standard amongst other groups studying dopamine receptor activation in rat anterior pituitary cells (Keja et al., 1992; Nussinovitch & Kleinhaus, 1992).

(iii) ATP has also been shown to increase the stability of recordings, reducing the effects of whole cell dialysis, or ‘wash out’ (Forscher & Oxford, 1985).

(iv) The inclusion of nucleotides in the intracellular solution is a routine procedure in Prof. Dolphin’s lab when investigating the G-protein modulation of expressed currents in the COS-7 cell line (a cell line that I also used in this study). Hence direct comparisons between my research and the research performed in the lab would be possible by using a similar intracellular
solution. In addition, Prof. Dolphin's lab routinely working on COS-7, HEK 293 and NG-108 cells had never observed such aberrant apparent inward rectification as was displayed by the GH4C1 D2 cell line currents. Therefore, amongst these cell lines, only the GH4C1 D2 cells may exhibit the problematic contaminating current.

(v) During my initial experiments in the HEK 293 cells (transient VDCC transfections and the HEK 293 αT cells) the inclusion of nucleotides did not appear to induce the erroneous leak subtraction. Therefore inclusion would allow further comparisons between my own research in different expression systems (HEK 293 and COS-7), and again with research performed in Prof. Dolphin's lab.

These reasons validated the use of a low Cl\(^-\) intracellular solution supplemented with nucleotides (ATP and GTP). Therefore, this low Cl\(^-\) based intracellular solution was used for all subsequent whole cell patch clamp experiments in HEK 293 and COS-7 cell lines.

### 3.3.1.2 Amphotericin-B perforated patch recording solution was chosen for perforated patch recordings

In consideration of the similarity of the amphotericin-B and nystatin perforated patch pipette solutions, identical except for the antibiotic utilised for membrane perforation, it is surprising how different the IV relationships appear (see Figure 3.6A). As mentioned in the results, the variation in \(I_{Ba}\) (density) maybe due to disparity between the whole cell capacitance (\(C_m\), 13.3 ± 2.6 pF and 24.2 ± 3.6 pF for amphotericin-B and nystatin respectively). This difference in \(C_m\) appears to be the cause of the apparent difference in \(I_{Ba}\) density, since the \(I_{Ba}\) before calculating the \(I_{Ba}\) density in each recording condition are not significantly different (-180 ± 27 pA for nystatin recordings and -158 ± 37 pA for amphotericin-B). The recordings with nystatin would appear to be the more unusual and possibly aberrant of the two \(C_m\) values.
measured for the permeabilising antibiotics: amphotericin-B recordings have a mean $C_m$ more closely resembling the mean $C_m$ values measured for all the other IV relationships recorded in the whole cell patch clamp configuration ($15.8 \pm 0.6 \text{ pF, } n = 69$). It is not clear why there was an increased mean $C_m$ value when using nystatin. These recordings were performed early in the Ph.D. experiments at the MSD laboratories in Harlow, though inexperience or amplifier errors do not appear to be suitable explanations for the apparently aberrant nystatin $C_m$ measurements, since whole-cell recordings made at the same time did not give significantly different $C_m$ measurements ($17.2 \pm 1.2 \text{ pF, } n = 20$). It was observed that the nystatin intracellular solution would often appear to be cloudy. This cloudy appearance was likely to be due to problems with the solubility of nystatin, resulting in nystatin re-crystallising within the patch-pipette. As a result findings with nystatin may be less reliable.

The IV relationship when recording with nystatin was depolarised in nature (peak $I_{Ba}$ occurs at a voltage step of 0 mV and the $V_{SO(spc)} = -14 \text{ mV}$), when compared to typical IV relationships observed in the whole cell patch clamp configuration (see Table 3.1). The IV relationship with amphotericin-B, however, was more typical in nature showing peak $I_{Ba}$ density ($12.9 \pm 2.8 \text{ pA.pF}^{-1}$, at $-10 \text{ mV}$) and $V_{SO(spc)} (-27.7 \text{ mV})$ more closely resembling the values observed when using the whole cell patch clamp technique. Again, a clear explanation for the different recordings made with nystatin was not obvious. Amplifier readings during nystatin perforated patch recordings, such as the series resistance ($R_s = 19.8 \pm 1.5 \text{ M}\Omega, n = 10$), did not suggest any irregularities that may have explained the IV relationship difference. In addition cell culture conditions cannot be used to explain the depolarised IV with nystatin, since the ten cells recorded were from two separate cell cultures (MSD and UCL, both originating from MSD frozen stocks, with recordings made at MSD) and the parameters of peak current density, $C_m$ and $R_s$ did not exhibit any significant difference between the two cell populations.
In addition to the more typical $C_m$ values and IV relationship parameters, amphotericin-B also provided a more practical cell membrane permeabilising agent. Using amphotericin-B, complete cell membrane permeabilisation was usually achieved in <5 min (compared to >7 min for nystatin) and was an effective membrane permeabilising agent for up to an hour after formation of the solution (whilst the permeabilising ability of nystatin solutions declined rapidly after 30 min). As a result of these considerations, the amphotericin-B perforated patch clamp intracellular solution was used for further experimentation.

3.3.2 RT-PCR of VDCC subunits in the GH4C1 D2 cell line

The RT-PCR (see Figure 3.7) detected the presence of mRNA for the VDCC $\alpha_{IA}$, $\alpha_{IC}$, $\alpha_{ID}$, $\alpha_{IE}$ subunits (no $\alpha_{IB}$ was detected) and $\beta_{1,3}$ subunits (but not $\beta_4$). In a study on the closely related GH3 clonal cell line, an attempt at detecting $\alpha_{IA-D}$ subunits was made using the ribonuclease protection assay: in support of my data $\alpha_{IA}$, $\alpha_{IC}$ and $\alpha_{ID}$ were detected and once again the $\alpha_{IB}$ was not found to be present (Lievano et al., 1994). The strong detection of the mRNA for the $\alpha_{IC}$ and $\alpha_{ID}$ subunits supports the assertion that a large proportion of the GH4C1 D2 VDCC current is the DHP-sensitive L-type current. The absence of the $\alpha_{IB}$ subunit (the main pore forming subunit in the HVA N-type channel; Williams et al., 1992a; Brust et al., 1993; Fujita et al., 1993; Stea et al., 1993) suggests that the N-type VDCC does not form part of the HVA VDCC population in the GH4C1 D2 cell line. The remaining HVA current that is blocked by Cd$^{2+}$ may be formed from the $\alpha_{IA}$ and $\alpha_{IE}$ subunits. However caution is needed in drawing conclusions from these data, because the RT-PCR only detects the presence of cytosolic mRNA: translation, post translational modifications and membrane translocation all need to be carried out for functional protein expression.

The contamination of the RT-PCR results by genomic DNA must also be considered. The contamination by genomic DNA is expected to be unlikely for two reasons. Firstly, the GITC method used (see section 2.5.1) has two chloroform and phenol extraction steps in
which small soluble total RNA are removed from larger, insoluble moieties (such as proteins, and including genomic DNA), each followed a 13 000 x g centrifugation. Secondly, if these mRNA purification steps are breached by contaminant genomic DNA, then the genomic DNA amplification products are likely to be significantly bigger than the predicted mRNA amplification products, due to the large numbers of introns found frequently throughout all VDCC subunits. For example, the forward primer for the $\alpha_{1C}$ VDCC subunit lies on exon 44 (human chromosome GenBank accession number z26264) and the reverse primer lies on exon 47 (accession number z26284); thus the amplification product resulting from genomic DNA amplification is expected to be several kilo-base pairs in length compared to the 574 base pairs expected of the $\alpha_{1C}$ mRNA.

These explanations suggest the possibility of genomic DNA contamination is low. To ensure genomic DNA was not present a negative control was performed in parallel on a sample of GH4C1 D2 cell line mRNA extract in which the RT step was omitted. Following the PCR amplification in this negative control, no amplification products were detectable suggesting that no genomic DNA was present (data not shown). This routine negative control step was also applied to the RT-PCR performed in the HEK 293 and HEK 293 $\alpha_{1D}$ cell lines (see Figure 4.1 later).

3.3.3 Pharmacology

In most of the following experiments the high Cl intracellular solution with ATP/GTP was used. These experiments (like the pre-pulse facilitation experiments described below) were performed before the leak error was discovered. With hindsight, based on the arguments relating to the P/N subtraction errors presented above (see section 3.3.1.1), the low Cl intracellular solution would have been chosen. However, leak subtraction errors are unlikely to be affecting these evoked peak current time-course based experiments: due to the comparatively small voltage steps (from $V_{\text{hold}} = -80$ mV to $V_i = -10$ mV) the P/4 leak subtraction protocol used would have estimated the leak current by hyperpolarising steps to
97.5 mV. At such hyperpolarised potentials even when using the high Cl\textsuperscript{-} with ATP/GTP intracellular solution the leak currents are still linear (see Figure 3.5A). Thus the recordings, serendipitously, are accurate. As a precaution some of the key G-protein modulating experimental conditions were repeated using a low Cl\textsuperscript{-} intracellular solution: the same lack of GPCR modulation (see section 3.3.3.2, below) of the calcium channel current remained.

3.3.3.1 Predominantly L-type current displayed in GH\textsubscript{4}C\textsubscript{1} D2 cell line

Previous studies using this GH\textsubscript{4}C\textsubscript{1} D2 cell line have shown that the current in these cells is predominantly L-type in nature, with approximately 90% of the \textit{I}\textsubscript{Ba} in these cells exhibiting block by 10 \textmu M nisoldipine (Seabrook \textit{et al.}, 1994). Likewise, in the present study a significant proportion of the \textit{I}\textsubscript{Ba} was shown to be inhibited by the L-type specific DHP nifedipine (under different conditions at \textit{V}\textsubscript{hold} = -80 mV approximately 60% of the \textit{I}\textsubscript{Ba} was inhibited; see Table 3.2 for a summary). Increased block by 10 \textmu M nifedipine at the lower \textit{V}\textsubscript{hold} of -60 mV (71 ± 1.7; \textit{n} = 3) was also observed in the closely related GH\textsubscript{3} clonal cell line (61 ± 4% at \textit{V}\textsubscript{hold} = -90 mV, \textit{n} = 5; 82 ± 5% at \textit{V}\textsubscript{hold} = -40 mV, \textit{n} = 3; see Lievano \textit{et al.}, 1994). This increased inhibition at more depolarised holding potentials has been shown to be a characteristic of DHP antagonist inhibition of L-type currents (Bean, 1984; Sanguinetti and Kass, 1984; Hamilton \textit{et al.}, 1986; though does not appear to be observed in the L-type \alpha\textsubscript{1D} channel currents of the HEK 293 \alpha\textsubscript{1D} cell line, see section 4.2.2.1). This increased inhibition is thought to arise due to a stabilisation of the channel in the inactivation state by DHP antagonists or an increased binding of the DHP antagonist ligand to the inactivated state of channels, a channel state which is favoured at depolarised potentials (Bean, 1984; Hamilton \textit{et al.}, 1986). In GH\textsubscript{4}C\textsubscript{1} D2 cells this increased DHP inhibition effect may also be partially due to an increase in the proportion of L-type current as a result of a decrease in the proportion of T-type LVA current, due to inactivation of this current component at the more depolarised \textit{V}\textsubscript{hold}. The rapid and reversible block by nifedipine and \textsuperscript{2}Ca\textsuperscript{2+} also rule out the possibility that drugs
are not reaching the cells or that onset is delayed by poor perfusion through the perfusion chamber.

**3.3.3.2 GH₄C₁ D2 cell line calcium channel currents do not display G-protein modulation**

*Investigation of GPCR pathways in GH₄C₁ D2 cells*

The lack of huD₂s receptor activation by Quin and DA was unexpected (see Figures 3.8A/C and 3.8B/E, and Table 3.2). In previous experiments with the same clonal cell line (Seabrook et al., 1994) almost half (9/21) the cells responded (23 ± 7% inhibition of peak current) to DA (10 μM) perfusion. Two changes that were made to recording solutions from those used by Seabrook et al. were the addition of GTP and ATP to the high Cl⁻ intracellular recording solution (see Methods, Table 2.4) and the main charge carrier in my experiments was 10 mM Ba²⁺ (as opposed to 5 mM Ca²⁺). These changes were present in the calcium channel current recording in which distinct inhibition during Quin perfusion was observed (Figure 3.1), performed at the start of this series of experiments. The addition of GTP and ATP intracellularly (explained fully in section 3.3.1.1) was in response to work by Lledo et al. (1990) in acutely isolated rat anterior pituitary cells which stated that using GTP-free intracellular conditions abolished the G-protein modulation effects of DA. Other workers studying the effects of dopamine receptor activation in rat pituitary cells also routinely included ATP and GTP in intracellular solutions (Nussinovitch and Kleinhaus, 1992; Keja et al., 1992). The change of charge carrier from 5 mM Ca²⁺ to 10 mM Ba²⁺ was to allow robust and stable calcium channel current recordings (reducing Ca²⁺-dependent run-down, Chad and Eckert, 1986).

One possible explanation for the loss of apparent huD₂s receptor activation is that although a stable cell line was created carrying the huD₂s DNA, during passaging the DNA may have "dropped" out of the GH₄C₁ D2 cell line genome, mutated or damaged, resulting in
no, reduced or aberrant, expression of the huD2s protein. The passage number did rise as high as passage 25, though with this possible drop-out effect in mind the vast majority of experiments were carried out at passages 9-16. Immunocytofluorescence was attempted using a human D2/3 receptor antibody: however due to poor specificity (cross-reactivity) of the antibody the presence or absence of the huD2s protein was not clarified (data not shown).

The immunocytofluorescence experiments were not explored further because it became apparent that the huD2s was not the only GPCR in the GH4C1 D2 cells that was not modulating the currents in response to agonist application. Activation of the endogenous sst2 and M4 GPCRs by SS-14 and Carb respectively also displayed no modulating effect upon the GH4C1 D2 cell calcium channel current (for examples see Figures 3.8C/F and 3.9; summarised in Table 3.2). This loss of effect in each of the GPCR pathways investigated suggests a different explanation for the lack of effect: the required G-protein complement to transduce the activated GPCR signal into current inhibition maybe missing or aberrant. This explanation would account for the loss across the three GPCR pathways investigated, and is more encompassing than the loss of the huD2s suggested above.

Thus with the exogenous huD2s and endogenous sst2 and M4 GPCRs all displaying a lack of G-protein modulation of the GH4C1 D2 cell line calcium channel currents, direct activation of the G-proteins was attempted using intracellular application of the GTP-γS. Intracellular GTP-γS does not require the activation of a GPCR pathway for channel modulation to be elicited, acting directly by activating the cytosolic G-proteins. However, even using this direct method of G-protein activation no characteristic modulation of the IV relationship was observed with and without intracellular GTP-γS (in both high and low Cl⁻ based intracellular solutions). This GTP-γS data, like the lack of response observed for the activation of each of the GPCRs, also suggests the GH4C1 D2 cells investigated were lacking a component in the G-protein transduction pathway. In a final attempt to observe G-protein
modulation characteristics on the \( \text{GH}_4 \text{C}_1 \) D2 cell line calcium channel currents pre-pulse facilitation experiments were performed (discussed in the following section).

\( \text{GH}_4 \text{C}_1 \) \( D2 \) cell line currents do not display pre-pulse facilitation in G-protein activating conditions

Earlier in this Discussion (section 3.3.1.1), when elaborating on the choice of the low Cl\(^-\) intracellular solution, it was stated that in high Cl\(^-\) intracellular solution at potentials hyperpolarised to \(-110\) mV non-linear leak currents were apparent (see Figure 3.5A). Unfortunately, many of the large depolarising pre-pulse experiments were performed using the high Cl\(^-\) and ATP/GTP (often substituted with GTP-\( \gamma \)S) intracellular solution before the erroneous leak subtraction effect was determined. These records will have inherent leak over-subtraction since voltage steps of 200 mV (from a \(-80\) mV holding potential up to the pre-pulse step of \(+120\) mV) with a \( P/4 \) leak subtraction protocol meant that leak subtraction test pulses were taken at \(-130\) mV: a potential at which Figure 3.5A clearly depicts non-linear leak currents. However, though quantitative analysis of these records would be unreliable, a qualitative assessment (pre-pulse effect or no effect) can be used. Under all the experimental conditions evaluated no pre-pulse facilitation was observed with the high Cl\(^-\) intracellular solution \( (n = 48; \text{see Table 3.3 for a summary}) \). On discovering the leak subtraction error all the G-protein activating conditions were re-examined using the low Cl\(^-\) intracellular solution, a solution that does not exhibit the non-linear leak currents at hyperpolarised potentials (see Figure 3.5B), and therefore will not cause over-leak subtraction to occur. In these low Cl\(^-\) intracellular conditions, again no pre-pulse facilitation was observed \( (n = 19; \text{Table 3.3}) \).
CHAPTER 4

PHARMACOLOGICAL AND G-PROTEIN MODULATION

STUDIES OF THE HUMAN, NEURONAL L-TYPE (α₁D, Caᵥ1.3)

VDCC IN HEK 293 CELLS
4.1 INTRODUCTION

This chapter describes the investigations into the VDCC currents exhibited in a human embryonic kidney (HEK) 293 cell line stably transfected with a human, neuronal L-type channel complex (α1D, α2δ-1 and β3a VDCC subunits). The data previously published on this HEK 293 α1D cell line comprised two abstracts written by the group that created the cell line (Hans et al., 1997; Brust et al., 1997). Therefore, the channels expressed in this cell line required further molecular and pharmacological characterisation. Subsequent experiments were carried to investigate whether this subtype of neuroendocrine L-type current was modulated by G-proteins, extending the theme that was initiated in the GH4C1 D2 cell line in Chapter 3.

Reverse-transcription polymerase chain reaction (RT-PCR) and immunocytofluorescence were used to confirm the VDCC subunit composition of the cell line. RT-PCR defined the presence of mRNA corresponding to the transfected VDCC subunits (α1D, α2δ-1 and β3a) in the HEK 293 α1D cell line, whilst α1D protein was detected by immunostaining with an α1D-specific antibody. Electrophysiological studies (whole cell and perforated patch clamp configurations) confirmed that the α1D expressing cell line exhibited typical L-type current characteristics. The currents evoked were high voltage activated (peak at +20mV) and showed little inactivation (long-lasting) in external Ba2+, whilst displaying rapid inactivation kinetics in external Ca2+. The L-type currents observed were inhibited by the 1,4 dihydropyridine (DHP) antagonists nifedipine and nicardipine, and were enhanced by the DHP agonist S-(-)BayK 8644. In contrast to human α1B N-type currents, α1D L-type currents were not modulated by activation of G-protein coupled pathways. Activation of endogenous somatostatin receptor subtype 2 (sst2) by somatostatin-14 (SS-14) or activation of transiently transfected rat D2 dopamine long receptors (rD2L) by quinpirole (Quin) had no effect. Direct activation of G-proteins by the non-hydrolysable form of guanosine 5'-triphosphate (GTP), guanosine 5'-0-(3-thiotriphospahite)(GTP-γS), also had no effect upon the α1D currents, as did guanosine-5'-0-(2-thiodiphosphate)(GDP-βS).
Furthermore, no modulation of the $\alpha_{\text{ID}}$ current by directly activating the cAMP dependent protein kinase-A (PKA) pathway using forskolin was observed. These data show that the biophysical and pharmacological properties of recombinant human $\alpha_{\text{ID}}$ L-type currents are similar to $\alpha_{\text{IC}}$ L-type currents, and these currents are also resistant to membrane-delimited (as opposed to modulation by second messengers, for instance; see sections 1.4.3 and 1.4.4) by G-proteins and $G_{\text{q/10}}$-linked G-protein coupled receptors.
4.2 RESULTS

4.2.1 Molecular characterisation of the HEK 293 α₁D cell line

4.2.1.1 RT-PCR of HEK 293 α₁D cell line VDCC subunit mRNA

Total RNA from a population of HEK 293 α₁D cells and from untransfected HEK 293 cells were amplified for specific VDCC subunits. These RT-PCR experiments were carried out for me by a technician in Prof. Dolphin's, Mrs. Julie Richards, and have been included here with her permission. The agarose gels of the amplified VDCC subunits are shown in Figure 4.1. RT-PCR amplification products from the HEK 293 α₁D cell line are displayed in Figure 4.1A. In previous experiments the primer specificity and reproducibility of amplification were confirmed using positive (specific subunit cDNA) and negative (sterile, distilled water) controls, though for clarity the agarose gels only display amplification products from test samples (total HEK 293 α₁D cell population RNA). A red tick in the top right corner defines bands of the correct predicted size (according to primer pair amplification product sizes see Methods, Table 2.2). For the HEK 293 α₁D cell line very clear, strongly UV fluorescent bands were detected for the stably transfected VDCC subunits α₁D, β₃ and α₂δ-1. In addition, very faint bands were detected for the α₁b, α₁e and β₁ [using primer pair β₁ (i)].

The RT-PCR amplification products arising from untransfected from HEK 293 cells are shown in Figure 4.1B. Surprisingly, several positive amplification bands were observed: correct sized migration products (again denoted by a red tick) were present for α₁A, α₁B, α₁D, β₁, β₃, β₄ and α₂δ-1. These data, however, should be interpreted with caution: some of these positive bands appear alongside multiple migration bands for the same subunit (e.g. the α₁B, β₃ and β₄ bands) which can indicate that erroneous amplification and possible misidentification of the mRNA present. Increased stringency between the primer pair and target cDNA sequence was attempted using higher melting temperatures during the PCR amplification thermal cycling, though the presence of these multiple migration bands were
Figure 4.1 Gel electrophoresis of RT-PCR products for VDCC subunits in HEK 293 cells.

For clarity, correct sized bands (see Table 2.2 for expected base pair sizes), where present (though may not be clearly apparent in the scanned gel), are indicated by a red tick at the top right corner of the specified band. Each gel had a λ BstE II digest (far left) and 100 base pair marker ladder (far right) electrophoresed alongside RT-PCR products to allow base pair size referencing. (A) RT-PCR products amplified from a population of HEK 293 α1D cells; and (B) HEK 293 cells.
never fully clarified (data not shown). In addition, as was mentioned previously with reference to the GH₄C₁ D2 cell line RT-PCR, the presence of specific mRNA species does not necessarily indicate the presence of functional protein. Endogenous calcium channel currents have been reported in HEK 293 cells (Berjukow et al., 1996). As a precaution untransfected HEK 293 cells were whole cell patch clamped, using the conditions used to patch clamp the HEK 293 α₁D cells, to determine the possible presence of endogenous currents: out of 11 cells 1 cell appeared to have a very small Iᵥ= -10 mV). To ensure that L-type calcium currents were not present but too small to observe or inactive, 3 µM S(-)-BayK8644 was perfused onto 9 of these cells (including the single cell displaying the <10 pA Iᵥ) and no effect was observed.

**4.2.1.2 α₁D protein immunostaining of HEK 293 α₁D cells**

To determine the presence of α₁D protein in the HEK 293 α₁D cell line, an α₁D specific antibody was applied to HEK 293 α₁D cells. HEK 293 cells (untransfected) were used as a negative control for this α₁D antibody immunoreactivity. In Figures 4.2A and B, HEK 293 and HEK 293 α₁D cells respectively were immunostained with the α₁D anitbody following permeabilisation. Strong α₁D immunoreactivity was observed in the HEK 293 α₁D cells (Figure 4.2B), but staining did not appear to be localised at the membrane as may have been expected. Some cells within this confocal microscope image may appear to be exhibiting some membrane specific staining (see the example cell highlighted by the arrow): however observation of numerous such images indicated that this was often due to large nuclei creating artefactual membrane-like staining. It is also apparent that the negative control HEK 293 cells, when permeabilised (Figure 4.2A), also show some weak immunoreactivity to the α₁D antibody. Clearer staining patterns were observed when cells were depolarised (and not permeabilised). Depolarisation of the cell membrane allows the α₁D epitope to be exposed exofacially, as explained in the Methods, section 2.6.1 (for further explanation see Wyatt et al., 1997). The negative control of HEK 293 cells under these depolarised conditions (Figure
Figure 4.2  Immunocytofluorescence of the HEK 293 and HEK 293 $\alpha_{1D}$ cell lines with the $\alpha_{1D}$ VDCC specific antibody.

In (A) HEK 293 cells and (B) HEK 293 $\alpha_{1D}$ were permeabilised using Triton X-100 detergent before fixing with paraformaldehyde and immunostained with the $\alpha_{1D}$ antibody. The scale bar in (A) represents 20 $\mu$m and is applicable to all the other confocal images here (B-D). In (C) HEK 293 and (D) HEK 293 $\alpha_{1D}$ cells were immunostained following depolarisation using solutions supplemented with 30 mM KCl (see Methods, section 2.6.3). The arrows depict example cells which appear to be displaying membrane specific immunostaining.
4.2C) did not show any obvious immunostaining for $\alpha_{1D}$. In contrast the HEK 293 $\alpha_{1D}$ cells under the same conditions (Figure 4.2D) show obvious $\alpha_{1D}$ immunoreactivity. Membrane specific binding may also be discerned in some cells (highlighted with arrows), though there were also cells in which the entire cell was stained, which may have been permeabilised during the immunocytofluorescence manipulation.

### 4.2.2 $\alpha_{1D}$ sensitivity to DHPs

The remaining experiments were performed using the whole cell and perforated patch clamp techniques to record calcium channel currents in the HEK 293 $\alpha_{1D}$ cells. Calcium channel currents were observed in 490 cells from 1129 cells in which recordings were attempted (that is, 43% of cells displayed observable currents). Of the cells that displayed the presence of calcium channel currents, approximately 120 cells (about 10% of the total cells attempted) were observed to provide sufficiently large and robust (> 30 pA), and acceptable values for voltage clamping parameters (see Methods, section 2.7.2, for the limits defined as acceptable), which allowed $I_{Ba}$ recordings.

#### 4.2.2.1 DHP antagonists: nifedipine and nicardipine

As expected the $\alpha_{1D}$ channels expressed in the HEK 293 $\alpha_{1D}$ cell line display sensitivity to DHP antagonists. The effects of 3 and 10 μM nifedipine are shown in the time course in Figure 4.3A, and the percentage inhibition of $I_{Ba}$ by 3μM nifedipine and nicardipine is shown in the bar chart in Figure 4.3B. The figure panels 4.3 C-F show the antagonist effect of nicardipine on an example IV relationship recorded from the same cell in control extracellular (Figures 4.3C and E) and nicardipine containing extracellular solutions (Figures 4.3D and F). Antagonism by this DHP antagonist was comparable to other studies on expressed L-type cloned channels (Williams et al., 1992b; Tomlinson et al., 1993). The 1,4-DHP antagonist block was also characterised by an increase in the inactivation kinetics of $I_{Ba}$ during the test depolarisation (+10 mV, $V_t$) which can be observed by comparing the inhibition at peak compared to the end of the 200ms test pulse (white and black circles
Figure 4.3 Inhibition of α_{1D} currents by the DHP antagonists nifedipine and nicardipine.

(A) Time course of currents measured at peak (open circles) and at the end (filled circles) of the 200 ms test pulse. Depolarising test pulses (V_t =+10 mV) were given every 30 s from a holding potential (V_{hold}) of −80 mV. Application of nifedipine (NIF) 3 and 10 μM are denoted by the horizontal bars. The inset shows example traces taken from the time course for control (CTRL, 10 mM Ba^{2+}) and for 3 and 10 μM NIF; these examples are taken from the time course at points 1, 2 and 3 respectively. The test pulse protocol is above these example traces. (B) Bar graph depicting mean current inhibition (%) at peak (white) and end of the 200 ms test pulse (black) for nifedipine (NIF, 3 μM, n = 7) and nicardipine (NIC, 3 μM, n = 6). (C-F) The affect of nicardipine (NIC) on an example IV relationship. (C) Example IV relationship traces evoked by performing the voltage pulse protocol above (C); for clarity only examples from this IV relationship for −40, −20, 0 and +20 mV are shown. (D) Example traces recorded from the same cell and using the same pulse protocol as in (C) after steady state inhibition by NIC (3 μM). The scale bars shown are also applicable to the traces shown in (C). (E) and (F) the IV relationships resulting from measuring IV protocol evoked recordings of CTRL (squares) and NIC inhibited (circles) I_{Ba} respectively, at peak (open symbols) and end (filled symbols) of the 200 ms IV test pulse.
respectively in Figures 4.3A and 4.3F, and white and black bars in Fig. 4.3B). Such an effect of 1,4-DHP antagonists to increase inactivation has been previously described (Lee & Tsien, 1983; Sanguinetti & Kass, 1984; Hamilton et al., 1986). Several groups have shown that block by DHP antagonists is voltage-dependent, inhibition being favoured at depolarised holding potentials (Sanguinetti & Kass, 1984; Bean, 1984; Hamilton et al., 1986). However, \( \alpha_{1D} I_{Ba} \) showed very similar inhibition by nifedipine at three different holding potentials \( (V_{hold} = -80, -50 \) and \(-30 \text{mV}) \). 3\( \mu \text{M} \) nifedipine inhibited the \( \alpha_{1D} I_{Ba} \) by approximately 60% (at peak) and 90% at (the end of the 200 ms test pulse) for each of the three \( V_{hold} \) values investigated. These DHP antagonist inhibition effects are summarised in Table 4.1 (see later).

### 4.2.2.2 DHP agonist: S(-)-BayK8644

The agonist S(-)-BayK8644 (3 \( \mu \text{M} \)) produced a typical enhancement of the current (325 \( \pm \) 25 % increase, \( n = 5 \), at \( V_t = +10 \text{mV} \) in 10mM \( \text{Ba}^{2+} \); 680 \( \pm \) 84 % increase, \( n = 14 \), at \( V_t = +10 \text{mV} \) in 20 mM \( \text{Ba}^{2+} \); Figure 4.4 and summarised in Table 4.1). The onset of enhancement was rapid (reaching steady state enhancement within 1-2 min of application, Figure 4.4A) and was also accompanied by a characteristic hyperpolarising shift in the IV relationship (Figure 4.4.B; see Hamilton et al., 1986; Williams et al., 1992b). To ensure this \( I_{Ba} \) enhancement was due to enhancement of the \( \alpha_{1D} \) calcium channel current and not the enhancement of an undefined endogenous calcium channel current population, whole cell patch clamp recordings were made in untransfected HEK 293 cells during perfusion of S(-)-BayK8644: a small current (< 10 pA) was observed in one cell, which displayed no enhancement of current, and eight cells displayed no current, and additional current was not revealed by S(-)-BayK8644 perfusion.
Figure 4.4 Sensitivity of $\alpha_{1D}$ currents to the DHP agonist S(-)-BayK8644.

(A) An example time-course of measured peak current (open circles) recorded with step depolarisations from $V_{hold}=-80$ mV to $V_t=+10$ mV, at 30 s intervals. A cartoon of the pulse protocol is shown above the inset example traces; numbers 1 (CTRL) and 2 (with BayK) denote the points on the time course at which these example traces were taken. The numbers beside traces denote the points along the time-course at which the example traces were taken. Application of S(-)-BayK8644 (3 µM) is denoted by the horizontal bar below time course. (B) An example cell showing a family of IV traces in control (20 mM Ba$^{2+}$; CTRL) and during S(-)-BayK8644 (3 µM; BayK) enhancement of the current. IV families were formed by depolarising from $-80$ mV to $-40$ mV, and in 10 mV increments up to $+60$ mV, every 5s (see pulse protocol cartoon below the CTRL family of traces). For clarity example traces are shown for currents measured by stepping to $-40$, $-20$, 0 and $+20$ mV only. In each condition peak current was measured and plotted as an IV relationship (lower panel; CTRL, filled squares; with BayK, filled circles) and fitted by a modified Boltzmann equation (Equation 1, see Methods, section 2.7.5).
Table 4.1 Summary of the DHP sensitivity calcium channel currents of HEK 293, HEK 293 $\alpha_{1D}$ and HEK 293 transfected with $\alpha_{1E}\text{long} \alpha_2\delta$-1$\beta_{3a}$.

Figures represent % mean±sem inhibitions (or enhancements for BayK) of peak $I_{Ba}$. In addition, where applicable, the increased inactivation observed with nifedipine and nicardipine is shown by showing figures at the end of the 200 ms depolarising test pulse (italicised). The following abbreviations are used: nifedipine, NIF; nicardipine, NIC; and, S(-)-BayK8644, BayK. The figures are for steady state inhibition/enhancement of the $I_{Ba}$ evoked by the standard pulse protocol ($V_{hold}$ as stated, $V_{t}=+10$ mV every 15 or 30 s; see Figure 4.3A). The unshaded sections are based on whole cell patch clamp recordings from HEK 293 $\alpha_{1D}$ cells. The last two shaded rows are $I_{Ba}$ recordings made in HEK 293 cells and HEK 293 cells transiently transfected with $\alpha_{1E}\text{long} \alpha_2\delta$-1$\beta_{3a}$. Inhibition figures with NIC in $\alpha_{1E}$ transfected cells were measured in response to 10 $\mu$M NIC, and are distinguished by *.

<table>
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<th>-30</th>
<th>-80</th>
<th>-100</th>
<th>-80</th>
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<td>BayK</td>
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<tr>
<td>conditions</td>
<td>3 $\mu$M</td>
<td>3 $\mu$M</td>
<td>10 $\mu$M</td>
<td>3 $\mu$M</td>
<td>3 $\mu$M</td>
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</tr>
<tr>
<td>10mM Ba$^{2+}$</td>
<td>63±5 (7)</td>
<td>62±7 (5)</td>
<td>56±7 (6)</td>
<td>90 (1)</td>
<td>---</td>
<td>30±4 (6)</td>
<td>325±25 (5)</td>
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<td>94±3 (5)</td>
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<td>97 (1)</td>
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<td>inhib'n @ 200ms</td>
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<tr>
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<td>741±159 (13)</td>
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<tr>
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<td>---</td>
<td>13±4 (9)</td>
<td>28±11 (3)*</td>
<td>63±5 (3)*</td>
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<tr>
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<tr>
<td>20mM Ba$^{2+}$</td>
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<td>---</td>
<td>---</td>
<td>32±9 (9)</td>
<td>52±11 (3)*</td>
<td>87±7 (3)*</td>
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<tr>
<td>HEK 293 with</td>
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<tr>
<td>inhib'n @200ms</td>
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4.2.3 Biophysical Characteristics

The steady-state inactivation of $\alpha_{1D}$ currents is shown in Figure 4.5. At test potentials up to $+30$ mV the $\alpha_{1D}$ currents did not fully inactivate and had a relatively depolarised $V_{50\text{act}}$ of $-13.4 \pm 1.8$ mV.

The inactivation kinetics at $V_i = +10$ mV were very slow in $\text{Ba}^{2+}$ (see Figures 4.3A, 4.4). Due to the lack of inactivation, longer depolarising $V_i$ were performed (1200-1600 ms). Single exponentials were fitted to the inactivating phase (e.g. the white line in the example trace shown in Figure 4.6A). The $\tau_{\text{inact}}$ was $439 \pm 50$ ms ($n = 4$). The inactivation kinetics with external $\text{Ca}^{2+}$ rather than $\text{Ba}^{2+}$ were far more rapid shown by the overlaid example traces in Fig. 4.6B.

Almost complete inactivation is observed over a 200ms depolarising pulse in $\text{Ca}^{2+}$ ($\tau_{\text{inact}} = 44.3 \pm 1.1$ ms, $n = 7$). Additionally, the peak current in 20 mM $\text{Ca}^{2+}$ was smaller than in 20 mM $\text{Ba}^{2+}$ ($I_{\text{Ca}}$ was $\sim$20% the size of $I_{\text{Ba}}$). The mean IV relationship in 20 mM $\text{Ba}^{2+}$ and 20 mM $\text{Ca}^{2+}$ exemplifies these differences (Figure 4.6C). This increased rate of inactivation when $\text{Ca}^{2+}$ was the charge carrier, due to calcium-dependent inactivation, is characteristic of L-type and $\alpha_{1C}$ channels (Chad et al., 1984; Chad & Eckert, 1986; Kalman et al., 1988).
Figure 4.6 IV Relationship and $\tau_{\text{inact}}$ of $\alpha_{1D}$ in 20 mM Ba$^{2+}$ versus 20 mM Ca$^{2+}$.

(A) Long test pulses (1200-1600 ms) were used to determine $\tau_{\text{inact}}$ in Ba$^{2+}$. An example of such a fit is shown for a trace recorded in 20 mM Ba$^{2+}$ with a +10 mV, 1200 ms test pulse, this gave a $\tau_{\text{inact}}$ of 436 ms. (B) Example traces in Ba$^{2+}$ and Ca$^{2+}$ obtained by depolarising the cells to $V_t = +10$ mV for 200 ms in each condition. By fitting a single exponential to the inactivation of the trace recorded in Ca$^{2+}$, a $\tau_{\text{inact}}$ of 39.1 ms was obtained. (C) Using the same I-V pulse protocol described in Figure 4.4B (depolarising from $V_{\text{hold}} = -80$ mV to $V_t = -40$ mV to +60 mV in 10 mV increments) IV relationships from 6 cells were measured in 20 mM Ba$^{2+}$ and then in 20 mM Ca$^{2+}$. Measurements for Ba$^{2+}$ (peak, filled squares; end of pulse, open squares) and Ca$^{2+}$ (peak, filled circles; end of pulse, open circles) were made and plotted against the $V_t$ to give the mean IV relationship.
The mean peak IV relationships for \( \alpha_{1D} I_{Ba} \) with 10 mM and 20 mM \( Ba^{2+} \) as the extracellular charge carrier are shown in Figure 4.7A. The peak \( \alpha_{1D} \) current with 10 mM \( Ba^{2+} \) was \( 5.0 \pm 0.7 \) pA.pF\(^{-1} \) (at \( V_t = +10 \) mV) and with 20 mM \( Ba^{2+} \) was \( 10.1 \pm 2.1 \) pA.pF\(^{-1} \) (at \( V_t = +20 \) mV). The mean IV relationship data points were fitted with a modified Boltzmann equation (Equation 1, see section 2.7.5): the parameters for these fits are given in the figure legend. Again the long-lasting current characteristic when \( Ba^{2+} \) is used as the charge carrier is highlighted by the example traces from a cell recorded in 10 mM \( Ba^{2+} \) (Figure 4.7B).

**Figure 4.7  IV relationship of \( \alpha_{1D} \) in 10 mM versus 20 mM \( Ba^{2+} \).**

(A) Using the whole cell patch clamp recording configuration, \( I_{Ba} \) were evoked using the I-V pulse protocol described in Figure 4.4B. The membrane was depolarised from \( V_{hold} = -80 \) mV up to +40 mV and subsequently to +60 mV in 10 mV incremental steps. Peak current densities (pA.pF\(^{-1} \)) were measured from recordings in 10 mM \( Ba^{2+} \) (open triangles) and 20 mM \( Ba^{2+} \) (filled squares) and plotted against the test step potential (\( V_t \)). A modified Boltzmann fit (Equation 1, see Methods, section 2.7.5) was made to each of the resulting IV relationships between -40 mV to +60 mV. From the fits the following parameters were calculated: for 10 mM \( Ba^{2+} \), \( g = 154 \) pS.pF\(^{-1} \), \( V_{rev} = 47.8 \) mV, \( V_{50(tec)} = -4.3 \) mV and \( k = 7.4 \) mV; for 20 mM \( Ba^{2+} \), \( g = 291 \) pS.pF\(^{-1} \), \( V_{rev} = 57.3 \) mV, \( V_{50(tec)} = 2.3 \) mV, \( k = 7.0 \) mV. (B) Example traces from an IV relationship recording made in 10 mM \( Ba^{2+} \). Traces are shown for voltage steps to -30, -20, -10, 0 and +10 mV.

![IV relationship of \( \alpha_{1D} \) in 10 mM versus 20 mM \( Ba^{2+} \).](image-url)
4.2.4 G-protein independent kinetics of $\alpha_{1D}$ currents.

4.2.4.1 G-protein activation: GPCR pathways

Having established the basic biophysical and pharmacological properties of $\alpha_{1D}$ currents, the currents were examined to determine whether they display G-protein modulation. Initially the modulation of the $\alpha_{1D}$ currents was compared to transiently expressed $\alpha_{1B}$ currents (known to be G-protein modulated), in HEK 293 cells following activation of endogenous somatostatin receptor subtype 2 (sst2). Application of somatostatin (SS-14, 100-500 nM, $n = 8$) had no effect upon the $\alpha_{1D}$ current (Fig. 4.8A and Table 4.2 – this table summarises all the GPCR pathway investigations in transfected and untransfected HEK 293 cells). In the example shown, the L-type nature of the currents was further exemplified by the block by

Figure 4.8 The lack of G-protein coupled receptor (endogenous sst2) modulation of $\alpha_{1D}$ currents: whole-cell recordings.

(A) A time-course of peak $\alpha_{1D}$ currents evoked every 30 s from $V_{\text{hold}} = -80$ mV to $V_t = +10$ mV (filled circles) in $\alpha_{1D}$ expressing cells in 20 mM Ba$^{2+}$. SS-14 (500 nM) and NIF (3 μM) application are denoted by the horizontal bars. No response was observed in response to SS-14 application (100-500 nM, $n=8$). The insets show overlapping example traces observed in control (CTRL, from point 1 in the time course), SS-14 (500 nM, from point 2). (B) Peak currents measured in $\alpha_{1B}2\beta_2-1\beta_3$ expressing cells ($V_{\text{hold}} = -80$ mV, $V_t = +20$ mV, every 30 s, filled squares). SS-14 (500 nM) application inhibited the current by $40 \pm 7\%$ ($n = 8$). Inset shows example traces in control (point 1) and during SS-14 inhibition (point 2).
nifedipine. Using the same endogenous receptor-based signalling pathway, application of SS-14 (500 nM) caused a rapid inhibition of \( \alpha_{1b} \) currents, observed in all cells tested (see Figure 4.8B and Table 4.2, mean inhibition approximately 40 \%, \( n = 8 \)). Due to the nature of whole-cell patch clamping, the internal contents of the cell can be disrupted, resulting in loss of normal signalling pathways within the cell. Such 'wash-out' effects can be minimised by using perforated-patch clamp techniques (Horn & Marty, 1988; Rae et al., 1991). To ensure that the loss of G-protein modulation was not due to such 'wash-out', we also used amphotericin-B perforated patches, however no modulation by SS-14 was observed of \( \alpha_{1d} \) currents (\( n = 5 \), Figures 4.9A). There was no evidence for inhibition at any potential examined, as depicted in the peak IV relationships in each of the conditions (control, SS-14 and wash, Figures 4.9B and C).

To test another G-protein coupled receptor pathway, the \( \delta_{2L} \) receptor was transiently co-expressed with GFP as an expression marker in the \( \alpha_{1d} \) cell line and also transiently co-expressed with \( \alpha_{1b} \alpha_{2}\delta-1 \beta_{3} \). However, application of the D2 agonist quinpirole (300 nM), had no effect upon \( \alpha_{1d} \) currents (\( n = 7 \), Figure 4.10A), although a clear effect was observed in 10/16 of the \( \alpha_{1b} \alpha_{2}\delta-1 \beta_{3} \) expressing cells (with a mean inhibition = 59 \( \pm \) 7 \%, \( n = 10 \); see the example cell in Figure 4.10B). This inhibition was greater than that produced by activation of the endogenous sst2 receptor, suggesting more efficient activation of this G-protein pathway by D2 receptors, but despite this, no inhibition of \( \alpha_{1d} \) currents was observed (Figure 4.10C).

The data from the GPCR pathway investigations are summarised in Table 4.2. In addition there is also data relating to SS-14 inhibition of transiently transfected \( \alpha_{1b} \alpha_{2}\delta-1 \beta_{2a} \) (different \( \beta \) subunit from the experiments described above). Table 4.2 also shows data from G-protein coupled pathways that were investigated by perfusion of forskolin activation of adenyly cyclase (see Figure 4.15) and a cocktail consisting of S(-)-BayK8644, SS-14 and Quin (see Figure 4.16).
Figure 4.9 The lack of G-protein coupled receptor (endogenous sst2) modulation of \( \alpha_{1D} \) currents: Amphotericin-B perforated patch recordings.

(A) Time-course of peak \( \alpha_{1D} \) currents evoked by depolarising from \( V_{\text{hold}} = -80 \text{ mV} \) to \( V_t = +10 \text{ mV} \) every 30 s. Application of somatostatin (SS-14, 500 nM) is denoted by the hatched box area. At points during this time course IV relationships were performed (control conditions, filled circle; SS-14, filled square; wash, filled triangle; see Figure 4.4B for the pulse protocol description). (B) Examples of families of traces evoked by the standard IV pulse protocol (depolarising from \( V_{\text{hold}} = -80 \text{ mV} \) to between -40 mV and +60 mV in 10 mV increments) for control, SS-14 and wash conditions (for clarity examples only from \( V_t = -40, -20, 0 \) and +20 mV are shown. (C) The IV relationships resulting from the measurement of peak current in each condition (control, filled circle; SS-14, filled square; wash, filled triangle).
Figure 4.10  The lack of G-protein coupled receptor (transiently expressed rD2L) modulation of α1D currents: whole-cell recordings.

(A) α1D expressing cells were co-transfected with rD2L receptor and GFP (expression marker); application of the D2 agonist quinpirole (Quin, 300 nM) had no effect on current (n = 7, black circle); test pulses (from $V_{h0}$= -80 mV to $V_I$ = +10 mV) were given every 15s. Traces during Quin (1) and control (2, CTRL) conditions are shown in the inset (overlapping). (B) Transient expression of α1Bα2S-1β3 and co-expression rD2L receptor: time-course of measured peak current [same pulse protocol as in (A) except $V_I$ = +20 mV, black square]: application of Quin (300 nM) inhibited $I_{Ba}$ in 10/16 cells tested. Example traces during CTRL (1) and Quin (2) application are shown in the inset. (C) Bar graph showing % inhibition (mean ± sem) for SS-14 application on α1D and α1B currents (first two columns), and for Quin on α1D and α1B currents (additionally co-transfected with rD2L receptor; 3rd and 4th columns respectively).
Table 4.2 Summary of the G-protein inhibition of \( I_{Ba} \) by GPCR agonists in HEK 293 \( \alpha_{1D} \) and HEK 293 cells transfected with \( \alpha_{1B} \), \( \alpha_{2\delta} \)-1 \( \beta_{3a} \).

The figures (where applicable) denote the mean±sem % inhibition of peak \( I_{Ba} \) on perfusion of the designated GPCR agonists. The following abbreviations were used: somatostatin-14, SS-14; forskolin, Forsk; quinpirole, Quin; and, a cocktail consisting of 3 \( \mu \)M S(-)-BayK8644, 500 nM SS-14 and 300 nM Quin. Italicised figures in brackets denote the \( n \) for each set of experiments. Unless stated all experiments were recordings made using the whole cell patch clamp technique. Recordings from HEK 293 \( \alpha_{1D} \) cells are unshaded, whilst recordings from HEK 293 cells transiently transfected with \( \alpha_{1B} \), \( \alpha_{2\delta} \)-1 \( \beta_{3a} \) are shaded in grey. In some of the experiments cells were additionally transfected with exogenous rD2L receptor (+ rD2L).

<table>
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<tr>
<th>Recording Conditions</th>
<th>SS-14 100 nM</th>
<th>SS-14 200 nM</th>
<th>SS-14 500 nM</th>
<th>Forsk 3 ( \mu )M</th>
<th>Forsk 10 ( \mu )M</th>
<th>Quin 300 nM</th>
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<tbody>
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<td>NO EFFECT (1)</td>
<td>NO EFFECT (5)</td>
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<td>NO EFFECT (4)</td>
<td>---</td>
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<tr>
<td>Perforated patch</td>
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<tr>
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<tr>
<td>+ rD2L</td>
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<td>NO EFFECT (7)</td>
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<td>20mM Ba(^{2+})</td>
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<td>---</td>
<td>59±7 (10/16)</td>
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<tr>
<td>HEK 293 with ( \alpha_{1B}\alpha_{2\delta} ) ( \beta_{3a} )+ rD2L 20mM Ba(^{2+})</td>
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<td>---</td>
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<td>59±7 (10/16)</td>
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4.2.4.2 G-protein activation: direct interaction using GTP-γS and GDP-βS

The GTP analogue GTP-γS can be used as a more direct way of activating G-proteins since it is non-hydrolysable and leads to their sustained activation, interfering with the normal constant cycle of GTP binding and hydrolysis by the Gα subunit GTPase activity (see section 1.4.1)(Dolphin et al., 1989; Kasai & Aosaki, 1989). Conversely, the GDP analogue GDP-βS can be used to block G-protein activation.

IV relationships of α1D currents in the presence of GTP-γS and GDP-βS

G-protein modulation of calcium currents can be also identified by a decrease in the current amplitude and a depolarising shift of the IV relationship with intracellular GTP-γS, whilst opposite effects (increase in current amplitude and hyperpolarising shift of the IV relationship) are seen with GDP-βS (due to removal of tonic G-protein modulation).

However, in the HEK 293 α1D cell line no significant difference was observed in the IV relationships across the G-protein activating conditions (control, n = 21; +GTP-γS, n = 23; +GDP-βS, n = 17; Figure 4.11).

Pre-pulse facilitation of α1D current does not alter with GTP-γS and GDP-βS

The existence of tonic modulation was examined by pre-pulse facilitation, using intracellular GTP-γS or GDP-βS. Figure 4.12A depicts the ratio of current in the absence (noPP) or immediately following (+PP) a large depolarising prepulse (+PP/noPP ratio) for control (CTRL, grey columns and associated example traces), with 100µM GTP-γS (black columns and associated example traces) and with GDP-βS intracellularly (white columns). It can be seen in both the histogram and also in the example traces relating to these +PP/noPP ratios (Figure 4.12A), that there was a small degree of facilitation (+PP/noPP ratio > 1) in all of the intracellular conditions. In addition, the activation time to 90% peak (tt90%) was shorter for all +PP than noPP currents (Fig. 4.12B). However, the magnitude of pre-pulse facilitation, and the activation tt90%, was unaltered by inclusion of GTP-γS or GDP-βS.
Figure 4.11 IV relationships of α₁D currents with intracellular GTP-γS or GDP-βS displays no characteristic G-protein modulation.

Currents were elicited by an IV relationship voltage step protocol (see Figure 4.4B for a description). The peak current densities were measured and meaned from recordings with different intracellular conditions. Control intracellular solution recordings were depicted with grey squares \((n = 21)\); with 100 µM GTP-γS supplementation (black triangles, \(n = 17\)); and with 2 mM GDP-βS (white triangles, \(n = 23\)). Modified Boltzmann fits (Equation 1, see Methods, section 2.7.5) were made to each set of data points: control fit (dashed line), \(g = 291 \text{ pS.pF}^{-1}\), \(V_{rev} = 57.3 \text{ mV}\), \(V_{so} = 2.3 \text{ mV}\), \(k = 7.0 \text{ mV}\); GTP-γS fit (black line), \(g = 374 \text{ pS.pF}^{-1}\), \(V_{mv} = 52.0 \text{ mV}\), \(V_{so} = 7.4 \text{ mV}\), \(k = 8.0 \text{ mV}\); and GDP-βS fit (dotted line) \(g = 326 \text{ pS.pF}^{-1}\), \(V_{mv} = 47.1 \text{ mV}\), \(V_{so} = 0.8 \text{ mV}\), \(k = 6.8 \text{ mV}\).

In comparison, in recordings made from cells transfected with α₁D channels, there was no evidence of pre-pulse facilitation using control intracellular pipette solution. However, following direct activation of G-proteins with GTP-γS (Figure 4.12C) there was a marked facilitation of the +PP current compared to the current without a PP. Under these recording conditions, the \(t_{pp90}\%)\ was also greater in the noPP current than in the current preceded by a prepulse, whereas in control conditions this was not apparent (Figure 4.12D).
Figure 4.12 Lack of G-protein modulation of α₁D via GTP-γS and GDP-βS.

The following shading is used in all histograms: control intracellular (CTRL, grey), with 100 μM GTP-γS (+GTP-γS, black) and with 2 mM GDP-βS (+GDP-βS, white). (A, C) Using the pulse protocol depicted in the top right (in which the test pulse was applied either preceded (+PP) or not (noPP) by a 75 ms prepulse to +120 mV, the measurements of (+PP/no PP) ratio were measured in CTRL, +GTP-γS and +GDP-βS for cells expressing α₁D (A) and α₁B (C). The +PP/noPP ratios were calculated by measuring the values of I_{ES} at 20 ms after the start of the test pulse. Figures beneath columns denote the numbers of experiments. Statistical significance was determined by using an unpaired Student’s t-test (**p<0.01) between CTRL and experimental conditions.

Example traces for noPP and +PP in α₁D and α₁B with CTRL and +GTP-γS intracellular conditions are shown above A and C, respectively. (B, D) Using the same cells used for the +PP/noPP determination in (A), the ttp90% was measured for both sets of currents. The ttp90% values were measured by determining the maximum current amplitude, and measuring the time at which the current reached 90% of its maximum amplitude. Statistical significance was determined using a paired Student’s t-test between the ttp90% for noPP (-) and +PP (+) currents, for each of the conditions (*p<0.05, **p<0.01).
The pre-pulse facilitation decay kinetics of the \( \alpha_{1D} \) current

The experiments displayed in Figure 4.13 investigated the pre-pulse facilitation duration (or decay of pre-pulse facilitation) of \( \alpha_{1D} \) currents recorded using control intracellular solution. In order to define this effect, only cells \(( n = 5 )\) that displayed distinct and obvious pre-pulse facilitation were investigated \(( I_{Ba} \text{ displaying } +\text{PP}/\text{noPP ratio} > 1.3 )\). Figure 4.13A shows the pulse protocol used to investigate this characteristic: the duration \(( \Delta t \text{ ms} )\) between the +PP current and the +120 mV pre-pulse was sequentially increased from 4.8 ms to 72 ms in 4.8 ms increments. Thus, a family of 15 traces with increasing time-lapse between the +120 mV pre-pulse and the noPP current were recorded in this fashion, though for clarity only the 1st, 7th and 15th of these traces are shown as examples in Figure 4.13A. The +PP/noPP ratio was calculated for each of these 15 traces, and the mean for each was plotted against the duration between the +120 mV pre-pulse and the noPP current \(( \Delta t \text{ ms} )\), shown in Figure 4.13B. The +PP/noPP ratio decays with a time constant of 43.5 ± 20.7 ms. A similar rapid off-rate of the pre-pulse facilitation effect was observed when the mean ttp_{90\%} measurements were fitted with a single exponential growth equation: the ttp_{90\%} for noPP currents were unaffected (hence a linear relationship) whilst the +PP ttp_{90\%} had an exponential growth with a time constant of 25.8 ± 9.2 ms.

Pre-pulse facilitation during \( \alpha_{1D} \) current enhancement by \( S(-) \)-BayK8644: effects with GTP-\( \gamma S \) and GDP-\( \beta S \)

\( \alpha_{1D} \) current enhancement by \( S(-) \)-BayK8644 was examined with control, +GTP-\( \gamma S \) and +GDP-\( \beta S \) intracellular solutions. Enhancement with control intracellular was 680 ± 84 % \(( n = 14, \text{ Table 4.2} )\); with GTP-\( \gamma S \) supplementation the enhancement was 741 ± 159 % \(( n = 13 )\); and for GDP-\( \beta S \) supplementation enhancement was 410 ± 57 % \(( n = 8 )\). Using a Student’s paired \( t \)-test a significant difference \(( p < 0.05 )\) was determined between % enhancement of
Figure 4.13  $\alpha_{1D}$ calcium channel currents that exhibit pre-pulse facilitation display rapid reinhibition kinetics.

(A) Using the pulse protocol shown the kinetics of pre-pulse facilitation in 5 cells was investigated. A standard pre-pulse facilitation protocol was adapted in which the duration ($\Delta t$ ms) between pre-pulse and the $+PP$ current was increased from 4.8 to 72 ms in 4.8 ms increments. For clarity the first ($\Delta t = 4.8$ ms), the seventh ($\Delta t = 33.6$ ms) and the last ($\Delta t = 72$ ms) evoked $I_{Ba}$ from an example cell are shown below the pulse protocol. (B) Measurements taken at 20 ms into the noPP and $+PP$ currents evoked using the protocol described in (A) were made to determine the $+PP$/noPP ratio at each of the 15 different durations for $\Delta t$, and plotted against this time duration (filled circles). The resulting curve was fitted with a first order exponential decay (see Equation 3, section 2.7.5). The time constant of decay was $43.5 \pm 20.7$ ms. (C) Using the same five cells as measured in (B), the $t_{tp}90\%$ (described in Figure 4.14) was measured for noPP (filled squares) and $+PP$ (open circles) currents with the varying $\Delta t$ ms duration. The resulting values were plotted against the duration of $\Delta t$, and data points were fitted with a first order exponential decay, as above. The dotted line was the fit to $+PP$ values with an exponential time constant of $25.8 \pm 9.2$ ms.
the α1D current in control intracellular and GDP-βS supplementation recordings, but non-significant when compared to the S(-)-BayK8644 enhanced recordings using intracellular GTP-γS supplementation.

The pre-pulse facilitation of these enhanced currents were investigated and displayed in Figure 4.14 (and the figures for these pre-pulse facilitation experiments are also summarised in Table 4.3). An example trace taken from a pre-pulse recording using the control intracellular solution; the pre-pulse voltage step protocol is depicted above the example I_Ba trace (Figure 4.14A). Figure 4.14B shows a small degree of pre-pulse facilitation was observed across all the intracellular conditions, whether G-protein activating (with GTP-γS) or non-activating conditions (with GDP-βS) were present. For comparison the +PP/noPP ratio for control extracellular and control intracellular was shown (far right) which was previously shown in Figure 4.12A. Below, in Figure 4.14C, the ttp90% are shown for each of the intracellular condition and using the same calcium channel current recordings as those depicted in Figure 4.14B. In a similar pattern to the ttp90% values observed under these three different G-protein activating conditions during control extracellular perfusion (Figure 4.12A), the ttp90% values for the +PP currents were all significantly smaller (p < 0.01) than ttp90% for the noPP currents. There was also significant difference between noPP in GTP-γS and GDP-βS (p< 0.01) and again between +PP in GTP-γS and GDP-βS (p< 0.05) current ttp90% values. The S(-)-BayK8644 enhanced currents differ from the control extracellular currents by displaying longer activation kinetics: significantly (p <0.01) greater values for ttp90% in both +PP and noPP currents were observed when compared to the values for control extracellular currents (the control extracellular +PP and noPP ttp90% values are shown on the final two columns on the far right of Figure 4.14C).
Figure 4.14  The lack of G-protein modulation of $\alpha_{1D}$ via GTP-$\gamma$S and GDP-\(\beta\)S during current enhancement by \(S(-)\)-BayK8644.

The following shading is used in all histograms: control intracellular + \(S(-)\)-BayK8644 (BayK) perfusion (CTRL, grey), with 100\(\mu\)M GTP-$\gamma$S + BayK (+GTP-$\gamma$S, black), with 2mM GDP-\(\beta\)S + BayK (+GDP-\(\beta\)S, white) and CTRL intracellular without BayK perfusion (CTRL, hatched). (A) An example trace from a recording using control intracellular solution after $\alpha_{1D}$ current enhancement by BayK (3 \(\mu\)M). The pulse protocol used to evoke the current is shown above the trace. This is actually a member of a family of traces evoked according to the pulse protocol shown and described in Figure 3.9B. Measurements were taken from the voltage step potential 10 mV hyperpolarised to the potential which evoked the measured peak current (-10 or 0 mV for BayK enhanced currents). (B) Using this pulse protocol in which the test pulse was applied either preceded (+PP) or not (no PP) by a 112.5 ms prepulse to +120 mV, the measurements of (+PP/no PP) ratio were measured in CTRL, +GTP-$\gamma$S and +GDP-\(\beta\)S for cells expressing $\alpha_{1D}$ after steady state enhancement by BayK perfusion, and for comparison measurements taken from $\alpha_{1D}$ currents that have been recorded in CTRL extracellular solution are shown on the far right (hatched). The +PP/no PP ratios were calculated by measuring the values of $I_{Ba}$ at 20 ms after the start of the test pulse. Figures beneath columns denote the numbers of experiments. (C) Using the same cells used for the +PP/no PP determination in (B), the ttp\(_{90\%}\) was measured for both sets of currents. The ttp\(_{90\%}\) values were measured by determining the maximum current amplitude, and measuring the time at which the current reached 90\% of its maximum amplitude. Statistical significance was determined using an unpaired Student’s t-test between the ttp\(_{90\%}\) for noPP (-) and +PP (+) currents, for each of the conditions (*p< 0.05, **p< 0.01). In addition a significant difference between the noPP ttp\(_{90\%}\) for GTP-$\gamma$S and GDP-\(\beta\)S (**p < 0.01) and the +PP ttp\(_{90\%}\) values for GTP-$\gamma$S and GDP-\(\beta\)S (*p< 0.05) was observed.
The +PP/noPP ratio data used in Figures 4.12 and 4.14 are summarised below in Table 4.3.

In addition the +PP/noPP ratios for α1B transfected cells co-transfected with the rD2L receptor are shown in this table. Pre-pulse inhibition was observed in control 20 mM Ba\textsuperscript{2+} extracellular (+PP/noPP ratio $<$1). During perfusion of extracellular solution containing Quin G-protein pre-pulse facilitation was observed (+PP/noPP ratio approximately 1.7). These +PP/noPP ratios in control and Quin G-protein modulating conditions observed in the α1B + rD2L transfected cells were significantly different (p $<$0.05).

Table 4.3 Summary of the pre-pulse facilitation ratio (+PP/noPP ratio) for HEK 293 α1D and HEK 293 cells transfected with α1B α2δ-1 β3a.

+PP/no PP facilitation ratios were calculated from I\textsubscript{Ba} evoked by performing pulse protocols as described in Figures 4.14 and 4.16. The test pulse depolarisation (V\textsubscript{t}) at which measurements were made was at a potential 10 mV hyperpolarised to the potential eliciting the peak I\textsubscript{Ba}. For HEK 293 α1D I\textsubscript{Ba} recordings (no shading) this was routinely V\textsubscript{t}=0 mV (V\textsubscript{r}= -10 mV during BayK enhancement of I\textsubscript{Ba}) and for HEK 293 cells transfected with α1B α2δ-1 β3a (grey shading) was V\textsubscript{t}= +10 or +20 mV. Measurements of I\textsubscript{Ba} for +PP currents and noPP currents were made at 20 ms into the test depolarisation for each of these currents. The final two rows display figures for HEK 293 cells transfected with α1B α2δ-1 β3a and additionally transfected with the rD2L receptor.

<table>
<thead>
<tr>
<th>Recording Conditions</th>
<th>+PP/noPP Ratio</th>
<th>+PP/noPP Ratio + BayK</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mM Ba\textsuperscript{2+}</td>
<td>1.11 (2)</td>
<td>---</td>
</tr>
<tr>
<td>20mM Ba\textsuperscript{2+}</td>
<td>1.22±0.08 (17)</td>
<td>1.40±0.12 (10)</td>
</tr>
<tr>
<td>20mM Ba\textsuperscript{2+} +100μM GTP-γS</td>
<td>1.15±0.07 (8)</td>
<td>1.38±0.16 (7)</td>
</tr>
<tr>
<td>20mM Ba\textsuperscript{2+} + 2mM GDP-βS</td>
<td>1.15±0.08 (8)</td>
<td>1.13±0.10 (6)</td>
</tr>
<tr>
<td>HEK 293 with α1B α2δ-1 β3a</td>
<td>0.93±0.08 (7)</td>
<td>---</td>
</tr>
<tr>
<td>HEK 293 with α1B α2δ-1 β3a 20mM Ba\textsuperscript{2+}</td>
<td>2.73±0.06 (5)</td>
<td>---</td>
</tr>
<tr>
<td>HEK 293 with α1B α2δ-1 β3a +100μM GTP-γS</td>
<td>0.88±0.08 (17)</td>
<td>---</td>
</tr>
<tr>
<td>HEK 293 with α1B α2δ-1 β3a + rD2L 20mM Ba\textsuperscript{2+}</td>
<td>1.72±0.63 (6)</td>
<td>---</td>
</tr>
<tr>
<td>HEK 293 with α1B α2δ-1 β3a + rD2L 20mM Ba\textsuperscript{2+} + Quin</td>
<td>1.72±0.63 (6)</td>
<td>---</td>
</tr>
</tbody>
</table>
4.2.4.3 G-protein activation: other forms of G-protein modulation

L-type channels can also be modulated via the PKA pathway (for reviews see Ismailov & Benos, 1995; Dolphin, 1999). The effects of the adenylyl cyclase activator forskolin (10µM) were also examined, however no effect was observed on the $\alpha_{1D}$ currents ($n = 4$). An example of such a recording during forskolin perfusion is shown in Figure 4.15: peak current and current kinetics were not modulated by the forskolin (see inset example traces).

---

**Figure 4.15** The $\alpha_{1D}$ current does not exhibit forskolin modulation.

An example time course of peak measured currents evoked by depolarising from $V_{hold} = -80$ mV to $V_i = +10$ mV at 30 s intervals. Application of 10 µM forskolin (denoted by the black bar) has no effect on the peak current size or current kinetics (overlapping example traces for control, 1, and forskolin, 2, are shown in the inset). This lack of effect by forskolin was observed in 4 cells.
In a recent study by Hernandez-Guijo et al. (1999), a form of voltage-independent G-protein modulation was observed of rat chromaffin cell L-type currents. Modulation was observed during perfusion of a cocktail of S-(-)BayK8644, and a number of receptor agonists including ATP, opioids with or without the additional inclusion of catecholamines. In Figure 4.16, G-protein modulation of the $\alpha_{1D}$ currents during S-(-)BayK8644 perfusion was investigated. The $\alpha_{1D}$ expressing cells were also transiently transfected with rD2L receptor, and after enhancement of the $\alpha_{1D}$ current by S-(-)BayK8644 (3$\mu$M), a cocktail of S-(-)BayK8644 (3$\mu$M), SS-14 (500nM) and quinpirole (300nM) was applied. No effect was observed of this cocktail of drugs ($n = 5$, Figures 4.16 A and B).

**Figure 4.16 $\alpha_{1D}$ does not exhibit sensitivity to a cocktail of BayK S-(-)8644, SS-14 and Quin.**

(A) Time course of measured peak currents in $\alpha_{1D}$ expressing cells ($V_{hold} = -80$ mV, $V_1 = +10$ mV for a duration of 200 ms every 15 s). Application of BayK S-(-)8644 (BayK, 3$\mu$M) and the receptor agonist cocktail are depicted by the horizontal bars. The cocktail consisted of BayK (3$\mu$M), SS-14 (500 nM) and Quin (300 nM) and had no effect upon $\alpha_{1D}$ currents ($n = 5$). (B) Example traces taken from the time course are denoted by numbers (1-4); left: CTRL (1) versus current enhanced by BayK (2); right: overlapping currents for BayK (3) versus cocktail (4) application.
4.2.5 Selectivity of 1,4-DHPs for L-type currents

Several reports suggest that the selectivity of some DHP antagonists for L-type channels may not be as complete as previously thought (Diochot et al., 1995; Furukawa et al., 1999). In Figure 4.17, the calcium channel currents resulting from transient expression of $\alpha_{1E}^{\text{long}}/\alpha_{2\delta-1}/\beta_{1b}$ are shown to exhibit inhibition by nifedipine and nicardipine (both at 10 $\mu$M). By comparing these inhibition values at the peak and end of the depolarising test pulse (for nifedipine, 13% inhibition at peak, 32% at the end of the test pulse, Figure 4.17A and Table 4.1), and for nicardipine (63% inhibition at peak, 87% at the end of the test pulse, Figure 4.17B and Table 4.1), the increased inactivation that was observed in the $\alpha_{1D}$ expressing cells is also apparent in these $\alpha_{1E}$ currents. Although, from the time course, the onset of inhibition is slower (Figure 4.17A and B), clear inhibition of $\alpha_{1E}$ is observed and suggests that care must be used when defining native L-type currents in cells showing a mixed population of channels by sensitivity to DHP antagonists (particularly for the more promiscuous effect displayed by nicardipine). It is

Figure 4.17 Dihydropyridine antagonist sensitivity of $\alpha_{1E}$ (with $\alpha_{2\delta-1}$ and $\beta_{1b}$) currents.

Time courses of $\alpha_{1E}$ current at the peak (open circle) and at the end of a 200 ms test pulse (filled circle), elicited by depolarising from $V_{\text{hold}}=-80$ mV to $V_i=+10$ mV, every 15s. (A) Application of nifedipine (NIF, 10 $\mu$M, horizontal bar) resulted in mean peak inhibition of $13 \pm 4\%$, and mean inhibition at the end of the test pulse of $32 \pm 9\%$ ($n=9$). Inset displays example traces during CTRL and NIF application. (B) Application of nicardipine (NIC, 10 $\mu$M, horizontal bar): mean peak inhibition was $63 \pm 5\%$ and at the end of the pulse was $87 \pm 7\%$ ($n=3$). Inset displays example traces during CTRL and NIC application.
therefore possible that in studies using pharmacological classification of calcium currents in native cells, currents due to expression of α1E have been partially mis-classified as L-type currents, and G-protein modulation may have been, at least in part, attributed to this current component.

4.3 DISCUSSION

4.3.1 Molecular identity of the HEK 293 α1D cell line

It is apparent from the RT-PCR of the RNA extracted from the HEK 293 α1D cell line that the transfected α1D, β3 and α2δ-1 VDCC subunits were present (Figure 4.1A). Positive amplification products were also detected for α1B, α1E and β1 in this cell line. In addition, RT-PCR of untransfected HEK 293 showed amplification products for α1A, α1D, α1E, β1, and α2δ-1 (Figure 4.1B); other subunits may (α1B, β3 and β4) be present, though were apparent alongside multiple amplification products, which may indicate erroneous non-specific amplification. RT-PCR experiments to determine the presence of β2 VDCC subunit were never performed successfully, and therefore whether this subunit is present or not remains unclear. The presence of several VDCC subunit mRNAs other than the desired α1D/β3/α2δ-1 subunit complement may suggest that the I_Ba recorded in the HEK 293 α1D cells was not as pure as presumed. However, the functional expression of other endogenous VDCC subunits appears to be extremely limited, exemplified by the lack of endogenous currents recorded. Whole-cell patch clamp recordings of untransfected HEK 293 cells showed that endogenous currents were rare and were very small when present (1 cell out of 11 had a peak I_Ba of approximately 10 pA). This observation is further supported by the lack of current enhancement on application of S(-)-BayK8644 (n = 9). In the HEK 293 α1D cell line
determination of endogenous calcium channel current are harder to determine since if present the calcium channel currents may be masked by the predominant L-type calcium channel current. However collating patch clamp data from the HEK 293 α_{ID} cell line suggests that endogenous calcium channel currents, if present at all, were rare and small, as was observed for the HEK 293 cells. The HEK 293 α_{ID} cell line calcium channel currents were routinely investigated with an IV relationship voltage pulse protocol at the start of a recording. This allowed a wide range of voltage steps to be investigated to reveal the possibility of calcium channel currents that may have been present with a different IV activation profile from the expected α_{ID} current. A significant proportion of the HEK 293 α_{ID} cells displayed no calcium channel current (639 cells out a total of 1129, 57%). The HEK 293 α_{ID} cells that did have a calcium channel current (43%) all showed an IV relationship with HVA IV relationship (generally peaking at V_t = +20 mV). In addition, of those recordings where DHP perfusion was performed (agonist or antagonists) all the cells responded (n = 57), indicating that the calcium channel current was predominantly, if not entirely, L-type in nature, when present (but see section 4.3.4).

The immunocytofluorescence with the α_{ID} specific antibody suggests that α_{ID} protein was present in the HEK 293 α_{ID} cells. Exposure of the α_{ID} target sequence by depolarisation (since the sequence is located on a transmembrane sequence between S5 and S6 in domain IV, Wyatt et al., 1997) showed clear immunostaining in HEK 293 α_{ID} cells (Figure 4.2D), with some cells showing clear membrane localisation of the immunofluorescence. The negative control HEK 293 cells showed no obvious immunostaining under such depolarising conditions (Figure 4.2C). However, when HEK 293 α_{ID} or HEK 293 cells were permeabilised before application of the α_{ID} antibody, staining was still apparent in the HEK 293 α_{ID} (Figure 4B) cells but, surprisingly, weak immunostaining was also observed in HEK 293 cells (Figure 4A). Thus, as expected α_{ID} protein was observed in the HEK 293 α_{ID} cells, though definitive conclusions must be
treated with caution based on the HEK 293 immunostaining in permeabilised cells. The unexpected HEK 293 staining has two possible explanations:

(1) Small amounts of α₁D protein were present in the HEK 293 cells. It is possible that the HEK 293 cells express some α₁D protein since RT-PCR did show the presence of α₁D mRNA in HEK 293 cells: however, functional expression of this protein seems unlikely based on the paucity of endogenous calcium channel currents, with no additional activation of dormant currents on perfusion of S(-)-BayK8644 (described above). Non-functional, or limited, expression of α₁D protein in HEK 293 cells would also allow explanation for the lack of staining during the depolarising conditions, since staining during depolarising conditions would require membrane specific localisation of the α₁D protein to elicit staining. The staining observed in HEK 293 cells during permeabilisation may indicate cytosolic α₁D protein was present (without translocation and functional expression at the membrane).

(2) The α₁D antibody produces some non-specific cross reactivity with other proteins. This second explanation would appear to be the less plausible of the two: a previous study using the same α₁D antibody showed specificity for the α₁D over the α₁C protein, and did not exhibit any non-specific cross reactivity in COS-7 cells (Wyatt et al., 1997).

The immuncytofluorescence could have been strengthened by additional negative controls. The rat pre-immunising peptide (see Methods, section 2.6.1) might have been pre-incubated with the rabbit anti-α₁D polyclonal antibody. Subsequent staining with the antibody would then be expected to display no staining, providing proof of the specificity of the antibody for its pre-immunising peptide; if staining is observed under these experimental conditions then the polyclonal antibody is not as specific as desired, showing cross-reactivity against other proteins other than the pre-immunising peptide sequence. Alternatively, cross reactivity may be arising due non-specific binding by the secondary (goat anti-rabbit IgG conjugated to biotin) or tertiary
(streptavidin fluorescein isothio-cyanate) conjugating agents. Repeating the experiments, omitting the primary anti-α1D antibody binding step, would control for this aspect of non-specific binding. These negative controls would provide data clarifying the possibility of non-specific binding, and therefore strengthen the case for the presence of small amounts of α1D protein in untransfected HEK 293 cells (explanation 1 above).

4.3.2 L-type current characteristics exhibited by expression of the human neuronal α1D clone

There are a number of key characteristics shown by the HEK 293 α1D cells investigated in this study that are acknowledged as being traits of L-type currents. Sensitivity to the DHP antagonists (nifedipine and nicardipine, Figure 4.3) and an agonist (S-(-)BayK8644, Figure 4.4) were observed. The degree of inhibition and enhancement by DHPs (summarised in Table 4.1) are comparable with other studies investigating the pharmacology of expressed cloned L-type channels (Williams et al., 1992b; Tomlinson et al., 1993). In addition, the increased inactivation observed during DHP antagonist application that has been reported previously for native cardiac L type channels (Lee & Tsien, 1983), was also apparent for the α1D currents. This effect of DHP antagonists on the inactivation kinetics was recently investigated by Handrock et al. (1999), who suggested that it is due to a second DHP binding site. However, care must be taken when using the characteristics of antagonism by DHPs, since, as shown in Figure 4.17, α1E channels also exhibit inhibition by DHP antagonists (Stephens et al., 1997), including the characteristic increase in inactivation. More selective pharmacological definition of L-type over α1E or other non-L type currents can be obtained by using low μM concentrations of nifedipine (rather than the more promiscuous nicardipine; an effect also observed in oocytes, Furukawa et al., 1999) or by agonism by S-(-)BayK8644, since α1E has previously been shown to be insensitive to S-(-)BayK8644 (Stephens et al., 1997). However, recent studies in HEK 293 cell expressing T-type (α1G)
channels showed the currents recorded in these cells were sensitive to nifedipine (10 \(\mu\)M) and also exhibited a small degree of enhancement during S(-)-BayK8644 (1 \(\mu\)M) perfusion (Lacinova et al., 2000). Though the degree of inhibition by nifedipine and enhancement by S(-)-BayK8644 of the T-type currents in the study were minimal, it is becoming increasingly apparent that the DHP sensitivity ascribed to the definition of L-type currents needs to be treated with care, and at least two or more L-type characteristics should be investigated in order to ensure accurate L-type current determination.

A further characteristic of DHP pharmacology was observed during S(-)-BayK8644 enhancement of the \(\alpha_{1D}\) calcium channel current, namely a slowed activation of the evoked current was observed at activating threshold potentials (V_t between -20 and 0 mV). This can be seen in the increased ttp90\% values for S(-)-BayK8644 compared to control extracellular (Figure 4.14). This slowed activation during S(-)-BayK8644 enhancement has been shown to be a characteristic of expressed \(\alpha_{1C}\) calcium channel currents (Grabner et al., 1996).

Many of the biophysical characteristics expected of L-type currents are also observed for the \(\alpha_{1D}\) currents. The IV relationship in Figure 4.6 shows the currents activating at about -20 mV and peaking at approximately +20 mV in 20 mM Ba\(^{2+}\), was also observed for other native L type channels, and \(\alpha_{1C}\) currents (Lacinova et al., 1995). They also exhibit the ion selectivity (Ba\(^{2+}\) > Ca\(^{2+}\)) typical of other native and cloned L type channels (Kalman et al., 1988; Pérez-García et al., 1995) with current density in 20 mM Ba\(^{2+}\) being approximately five times that seen for 20 mM Ca\(^{2+}\). The steady-state inactivation (V_{50\%\text{inact}} at -13.4 mV), is also comparable to other expressed L-type currents (Lacinova et al., 1995). The inactivation kinetics are also typical of 'long-lasting' L-type currents (Nowycky et al., 1985; Tomlinson et al., 1993; Ihara et al., 1995). In 20 mM Ba\(^{2+}\) little inactivation of \(\alpha_{1D}\) currents was observed over the 200 ms voltage test pulse used, whilst rapid and striking calcium-dependent inactivation was observed in 20 mM Ca\(^{2+}\).

Another characteristic of the \(\alpha_{1D}\) currents that correlates well with other studies of expressed \(\alpha_{1C}\) channels (Dai et al., 1999; Kamp et al., 2000) was the small but reproducible
facilitation following a large depolarising pre-pulse (see Figure 4.12A). This characteristic is discussed further in section 4.3.3. Such attributes are often indicative of G-protein modulation, however for the \( \alpha_{1D} \) current this effect was independent of G-protein modulation, as it was similar in the presence of GTP-\( \gamma \)S and GDP-\( \beta \)S.

As yet there are no biophysical characteristics or pharmacological tools that can differentiate between currents resulting from either native or expressed \( \alpha_{1C} \) and \( \alpha_{1D} \) channels. However, previous research has shown that the block by DHP antagonists is voltage dependent, with greater inhibition being observed when the holding potential is more depolarised. However, for the \( \alpha_{1D} \) currents no such voltage-dependence of block by DHP antagonists was observed, with similar block occurring (at both peak and at the end of the 200 ms test pulse) at all holding potentials used. Another aspect that may prove to be different is the \( \tau_{\text{inact}} \) of \( \alpha_{1D} \) currents in Ba\(^{2+} \). In a previous study examining the \( \tau_{\text{inact}} \) of \( \alpha_{1C} \) when co-expressed with \( \beta_3 \) in \textit{Xenopus laevis} oocytes (Soldatov \textit{et al.}, 1997) even slower rates of inactivation were observed (~1300ms). However, in general care must be taken in interpreting such results since expression system (oocyte versus HEK 293) and specific accessory subunit composition (particularly \( \beta \) subunits) will have effects upon this biophysical trait.

### 4.3.3 Lack of G-protein modulation of \( \alpha_{1D} \) currents.

**GPCR activation pathways**

The preceding biophysical and pharmacological studies clearly showed that the \( \alpha_{1D} \) currents display L-type current characteristics. The possibility of G-protein modulation of this L-type current was then examined either by activation of the endogenous sst2 receptors or by transient expression of another GPCR, the \( \text{rD2L} \) receptor. However, no modulation was observed of \( \alpha_{1D} \) currents via either pathway (Figure 4.8A and 4.10A). Using amphotericin-B perforated patch recordings, to reduce the cytosolic disruption observed with whole cell patch clamping, \( \alpha_{1D} \) currents were still resistant to G-protein modulation (Figure 4.9). To
ensure that the G-protein pathways were intact and capable of coupling to calcium channels in the HEK 293 cells, both the endogenous sst2 and the transiently expressed exogenous rD2L receptors were stimulated via their respective agonists in cells expressing α1b currents (which have been previously shown to be G-protein modulated, Toth et al., 1996). These positive controls showed obvious G-protein modulation (Figures 4.8B and 4.10B), confirming that modulation is possible by these pathways in HEK 293 cells. Furthermore, the modulation of the current was also investigated during application of S(-)BayK8644, since a G-protein modulation effect had been observed in native rat chromaffin L-type channels, selected by their response to S(-)BayK8644 (Hernandez-Guijo et al., 1999). However a combination of S(-)BayK8644, SS-14 and quinpirole did not produce any G-protein modulation of S(-)BayK8644-enhanced α1d currents co-expressed with rD2L (see Figure 4.16). These data and the lack of G-protein modulation of the α1d calcium channel current across the range of paradigms investigated are summarised in Table 4.2.

**G-protein activation by GTP-γS and GDP-βS**

Another method to examine G-protein modulation is to use the non-hydrolysable GTP and GDP analogues GTP-γS and GDP-βS. The advantage of using these guanine nucleotide analogues is that they act directly upon all G-proteins, producing maximal activation (in the case of GTP-γS) or complete lack of activation (GDP-βS). No G-protein modulation of the α1d calcium channel current was observed in IV relationships between control, with GTP-γS or with GDP-βS (Figure 4.11).

Further pre-pulse evidence for a lack of G-protein modulation was provided using a standard large depolarising (+120 mV) pre-pulse protocol to detect G-protein modulation, no GTP-γS-dependent effect was observed on α1d currents; yet the α1b currents do exhibit marked tonic G-protein modulation in these conditions (Figure 4.12 and Table 4.3). This lack of effect of GTP-γS and GDP-βS on pre-pulse facilitation was also present in S(-)BayK8644 enhanced currents (Figure 4.14); currents in these conditions also showed slowed activation, an L-type current characteristic (see section 4.3.2). A range of depolarising pre-
pulse protocols were investigated (data not shown), varying the amplitude (from +90mV to +120 mV) and duration (from 75 ms to 112.5 ms) of the depolarising pre-pulse, and also the time between the pre-pulse and the subsequent test pulse (from 4.8 ms to 72 ms); however, G-protein modulation was never observed.

**G-protein effects during S(-)-BayK8644 enhancement suggest a G-protein/αID channel interaction, but without typical G-protein inhibition**

Although no typical G-protein modulation was observed in the αID calcium channel current, it has been suggested that G-protein interaction with L-type channels may occur (Scott & Dolphin, 1987; Scott & Dolphin, 1988) since DRG neurons were prevented from exhibiting BayK8644 (racemic mixture) current enhancement when pre-treated with pertussis toxin (PTX). Exploring this G-protein interaction effect which appears to be manifested only during BayK8644 current enhancement, the stereoisomeric form S(-)-BayK8644 was used to enhance αID calcium channel current with different G-protein activating intracellular conditions. There was a significant difference (p <0.05) in enhancement during S(-)-BayK8644 enhancement of αID calcium channel current when recording in control (680 % enhancement) compared to GDP-βS supplemented (410 %, see Table 4.1) intracellular solutions. This result may suggest that tonic levels of G-protein activation may allow sufficient G-protein interaction with the αID channels to cause this difference in S(-)-BayK8644 current enhancement. However, this hypothesis would predict that a significant increase, compared to enhancement with GDP-βS recordings, would also be observed with GTP-γS supplementation. Though the mean enhancement with GTP-γS (741 %) was greater than with GDP-βS present, the variability of the enhancement with GTP-γS prevented the increase being statistically significant compared to the enhancement with GDP-βS.

Additional evidence for a possible G-protein interaction with the αID channel was observed in the ttp90% values during S(-)-BayK8644 enhancement (see Figure 4.14C): both the +PP current and noPP current ttp90% values were significantly different between GTP-γS and GDP-βS intracellular solutions. However, though these data indicate a possible G-protein interaction with the αID channel, the apparent interaction does not create characteristic G-
protein modulation since the activation kinetics in S(-)-BayK8644 enhanced currents with intracellular GTP-γS were shorter than those observed with GDP-βS (opposite to the effect observed in typical G-protein inhibition). Thus, further clarification is required to define the possibility of the α1D calcium channel and G-protein interaction, and also to define what the effect of this interaction may be, since typical G-protein inhibition of the channel current appears to be a highly unlikely effect of this interaction.

**α1D pre-pulse facilitation decay rate**

Pre-pulse facilitation in non-L type HVA VDCCs has been shown to be due to the temporary removal of activated Gβγ protein subunits bound to the VDCC α1 subunit. Therefore pre-pulse facilitation decay rate (also referred to as G-protein re-inhibition rate) is thought to relate to the re-binding of Gβγ subunits, manifested by the pre-pulse facilitation decay rate being altered according to intracellular Gβγ subunit concentration (Zamponi and Snutch, 1998; Stephens et al., 1998a). Due to the inevitable differences, for example in Gβγ concentration, between recording systems pre-pulse facilitation decay rates in Gβγ inhibited currents vary from approximately 10 to 110 ms (Zhang et al., 1996; Patil et al., 1996; Currie & Fox, 1997; Zamponi & Snutch, 1998). The decay rate for the α1D current (43.5 ms, see Figure 4.15) lies within these Gβγ based facilitation decay rates. However, the lack of current modulation by GTP-γS and GDP-βS suggests Gβγ subunits are not the cause of this α1D pre-pulse facilitation.

Pre-pulse facilitation has also been observed in cardiac L-type calcium channel currents due to modulation of the current by cAMP dependent PKA (Sculptoreanu et al., 1993). The pre-pulse facilitation decay in these phosphorylating conditions decayed bi-exponentially with time constants of 12 and 100 ms. Once again the α1D calcium channel current pre-pulse facilitation decay lies within these time constants. However, the pre-pulse facilitation observed in the α1D currents appears to be independent of cAMP dependent PKA modulation. The α1D calcium channel current was not modulated by forskolin (see Figure 4.15; though see the cautionary note, below). In addition, the pre-pulse facilitation resulting from PKA modulation reported by Sculptoreanu et al. (1993) displayed increasing +PP/noPP
ratios over a large range of pre-pulse durations (with time constants up to 8 s). In the \( \alpha_{1D} \) calcium channel current, pre-pulse facilitation showed no significant difference when using a pre-pulse duration of 75 ms and one of 112.5 ms (data not shown), suggesting the level of pre-pulse facilitation had plateaued at a pre-pulse duration <75 ms.

Two studies recently have suggested that in HEK 293 cells transfected with the cardiac (\( \alpha_{1C} \)) L-type \( \alpha_1 \) subunit, along with accessory subunits, display pre-pulse facilitation that is different from previously described facilitation forms (Dai et al., 1999; Kamp et al., 2000). The facilitation observed in these L-type currents was independent of G-protein modulation and cAMP dependent PKA phosphorylation. Many of the traits of this cardiac L-type pre-pulse facilitation were very similar to that observed in the studies presented here on the \( \alpha_{1D} \) calcium channel current. Kamp et al. (2000) showed that in \( \alpha_{1C} \) expressed channels the pre-pulse duration at which the effect was observed to plateau was rapid (\( \tau = 7 \) ms), and the pre-pulse facilitation decay was very similar to that observed in the \( \alpha_{1D} \) current (34.6 ms compared to 43 ms for \( \alpha_{1C} \) and \( \alpha_{1D} \) currents respectively). However, Dai et al. (1999) also showed that the pre-pulse facilitation effect observed in the cardiac L-type currents was abolished by co-transfection of the \( \alpha_2 \delta \) VDCC subunit, a subunit that was transfected into the HEK 293 \( \alpha_{1D} \) cell line. Thus, in order to fully reconcile the pre-pulse facilitation observed in the \( \alpha_{1D} \) calcium channel current with the facilitation observed in the \( \alpha_{1C} \) currents of these studies, further experimentation is required.

Lack of modulation by G_s-protein activation

G_s-protein mediated modulation of L-type currents was also investigated by activation of the G_s-adenyl cyclase pathway with forskolin. No effect of forskolin was observed (Figure 4.15). PKA modulation of channels has been shown to require A-Kinase Anchoring Proteins (AKAPs, Johnson et al., 1997). The presence of AKAPs was not examined in this study, though they are likely to be present since they are found in tsA-201 cells which are an HEK 293 derived cell line (Johnson et al., 1997). However, further study is needed since other studies have shown that PKA modulation of L-type currents in HEK 293 cells require AKAP co-transfection (Zong et al., 1995a; Gao et al., 1997).
CHAPTER 5

THE EFFECT OF THE AUXILIARY $\beta_{2a}$ SUBUNIT ON THE G-
PROTEIN MODULATION BY $G_{\beta\gamma}$ OF THE $\alpha_{1B}$ (N-TYPE) VDCC
EXPRESSED IN COS-7 CELLS
5.1 INTRODUCTION

In this third and final results chapter the $G_{\beta\gamma}$ G-protein modulation of transiently expressed $\alpha_{1B}$ channels and the effect of co-expressing the auxiliary $\beta_2a$ VDCC subunit was investigated in the COS-7 cell line. The experiments were performed in response to unusual results observed in Prof. Dolphin’s lab. Single channel recordings on COS-7 cells transiently expressing either $\alpha_{1B}$ alone or $\alpha_{1B}$ with $\beta_2a$ were performed by Dr. Alon Meir, and tonic G-protein inhibition of these two expressed channel compositions was attempted by co-expressing $G_{\beta\gamma}$ G-protein subunit. In COS-7 cells expressing $\alpha_{1B}/\beta_2a$ with $G_{\beta\gamma}$ typical G-protein modulation was observed. However, in COS-7 cells expressing $\alpha_{1B}$ (alone) with $G_{\beta\gamma}$ typical G-protein modulation effects were not observed (Meir et al., 2000). These results were unexpected since previous research had suggested that the presence of VDCC $\beta$ subunits reduced G-protein modulatory effects upon VDCCs (Roche et al., 1995; Campbell et al., 1995a; Bourinet et al., 1996).

To investigate this observation further, experiments described here were performed in COS-7 cells in which $\alpha_{1B}$ and $\alpha_{1B}/\beta_2a$ VDCC subunits were co-expressed with the rat D2long (rD2L) receptor. These different combinations of VDCC subunit expression with the rD2L ($\alpha_{1B}$/rD2L or $\alpha_{1B}/\beta_2a$/rD2L) allowed investigation of the phenomena that was observed in tonic inhibition of single channels to be attempted in whole cell patch clamp recordings using GPCR activated $G_{\beta\gamma}$. Extensive pharmacological and biophysical study of these channels was not performed since these channel aspects had previously been thoroughly investigated using this $\alpha_{1B}$ clone expressed in both Xenopus oocyte and COS-7 expression systems in Prof. Dolphin's lab (Page et al., 1997; Page et al., 1998; Dolphin et al., 1999). However, some basic channel biophysics were determined by IV relationship comparisons between $\alpha_{1B}$/rD2L and $\alpha_{1B}/\beta_2a$/rD2L expressing cells: increased calcium channel current amplitude and a hyperpolarising shift in the current activation was observed with $\beta_2a$ co-expression; these are characteristic effects of $\beta$ subunit co-expression (reviewed by Walker...
and De Waard, 1998). The N-type nature of the α₁B channels was also confirmed by strong inhibition by the N-type specific channel blocker ω-CgTx GVIA of calcium channel current in an α₁B/β₂/α₂L expressing cell. G-protein modulation by activation of the co-expressed rD₂L GPCR confirmed the observations that had been made by Meir et al. (2000). Activation of the rD₂L receptor by the dopamine receptor agonist quinpirole (Quin) displayed typical G-protein modulation of the calcium channel current in α₁B/β₂/α₂L expressing cells (observed in 13/14 cells). Typical G-protein effects that were observed included: calcium channel current inhibition; pre-pulse facilitation following a large (+120 mV) depolarising pre-pulse; depolarising shifts in the IV relationship; and slowed current activation during G-protein activation by Quin. In α₁B/β₂L expressing cells the G-protein modulation following Quin activation was significantly different to the modulation observed in the channels co-expressed with β₂L. Only 6/12 cells responded to Quin perfusion in the α₁B/β₂L expressing cells and the G-protein effects were less apparent in cells that responded. One explanation of the heterogeneity of response observed in the α₁B/β₂L expressing cells was contamination of the desired α₁B (alone) channels by endogenous COS-7 cell β subunit expression. This explanation is possible since the β₃ subunit was detected by RT-PCR on a population of COS-7 cells.

These whole cell patch clamp data confirm the observations made by Dr. Meir in single channel recordings on α₁B expressed channels with and without β₂L co-expression, namely that β subunit expression appears to be an integral part of the channel composition required for complete G-protein Gβγ modulation. Where α₁B channel modulation was attempted in the absence of VDCC β subunit co-expression the G-protein modulation of the resulting channel calcium channel current was limited and did not display typical characteristics expected of G-protein modulation.
5.2 RESULTS

5.2.1 $\alpha_{1B}$ channel characteristics expressed in COS-7 cells

$IV$ relationships for $\alpha_{1B}/rD2_L$ and $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells

IV relationships were performed with an extracellular solution containing 10 mM Ba$^{2+}$ as the charge carrier. The mean current density across a range of voltage steps ($V_i = -40$ to $+80$ mV) are shown for $\alpha_{1B}/rD2_L$ and $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells in Figure 5.1A. The co-expression of the $\beta_{2a}$ VDCC subunit resulted in a mean current density approximately three times greater than for $\alpha_{1B}$ alone channels ($14.5 \pm 7.9$ pA.pF$^{-1}$ compared to $4.5 \pm 1.7$ pA.pF$^{-1}$, though the effect was not statistically significant). The IV relationship was hyperpolarised by approximately 10 mV with $\beta_{2a}$ co-expression, which was apparent in the $V_{50(\infty)}$ ($+6.6$ mV with $\beta_{2a}$ and $+17.6$ mV with $\alpha_{1B}$ alone) and in the voltage step eliciting peak current amplitude ($+20$ and $+30$ mV with $\beta_{2a}$ and $\alpha_{1B}$ alone, respectively). The example current traces in Figure 5.1B show that calcium channel current in $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells displays a slow activation rate across the 100 ms voltage step. In comparison, $\alpha_{1B}/rD2_L$ expressing cells exhibited rapid activation rates, and also displayed inactivation over a 100 ms voltage step, as observed in the example traces shown. This slowing of activation and retardation of inactivation of $\alpha_{1B}$ calcium channel currents by $\beta_{2a}$ co-expression was recently described by Stephens et al. (2000) and was explained by an alteration of the $\alpha_{1B}$ channels by the presence of a $\beta$ subunit and particularly palmitoylation sites found on the $\beta_{2a}$ subunit. In addition, tonic G-protein modulation of the current in $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells may also add to the slowing of the current activation, since slowed activation kinetics are characteristic of G-protein modulated currents. This explanation is feasible since slowed current activation was shown to be present in control conditions and was removed by a large depolarising pre-pulse (see example traces, later, in Figures 5.3C and 5.4C, and also the larger $t_{tp90\%}$ value observed in currents without a pre-pulse which is significantly reduced following a pre-pulse, Figure 5.5B). Such tonic
Figure 5.1 IV relationships for calcium channel currents in COS-7 cells expressing $\alpha_{1B}\beta_2a/rD_{2L}$ and $\alpha_{1B}/rD_{2L}$.

(A) Peak calcium channel currents were measured using 10 mM Ba$^{2+}$ in the extracellular solution. The voltage step protocol used is described in (B). Mean recordings for $\alpha_{1B}\beta_2a/rD_{2L}$ (filled squares) and for $\alpha_{1B}/rD_{2L}$ (open circles) expressing cells are shown. A modified Boltzmann fit (Equation 1, see Methods, section 2.7.5) was made to each of the IV relationships. From the resulting fits, the following parameters were calculated: for $\alpha_{1B}\beta_2a/rD_{2L}$ expressing cells, $g = 324$ pS.pF$^{-1}$, $V_{rev} = +66.3$ mV, $V_{50(act)} = +6.6$ mV and $k = 5.5$ mV; for $\alpha_{1B}/rD_{2L}$ expressing cells, $g = 135$ pS.pF$^{-1}$, $V_{rev} = +66.5$ mV, $V_{50(act)} = +17.6$ mV and $k = 5.2$ mV. (B) The voltage pulse used to elicit the IV relationships in (A) is shown in the upper of three panels. The membrane voltage was stepped from $V_{hold} = -80$ mV to $-40$ mV and up to $+70$ mV in 10 mV increments. The duration of each voltage step was 100 ms. The lower two panels display example traces from an $\alpha_{1B}\beta_2a/rD_{2L}$ expressing cell (middle panel) and an $\alpha_{1B}/D_{2L}$ expressing cell (lower panel). The scale bar (middle panel) applies to both sets of current traces. For clarity only $I_{Ba}$ traces during the activating phase of the IV relationships (at $V_t = -10$, 0, +10, +20 and +30 mV) are shown.
inhibition, and hence current activation slowing, was not apparent in $\alpha_{1B}/rD2_L$ expressing cells. However, when $\alpha_{1B}/rD2_L$ expressing cells were divided into those that responded to Quin perfusion and those that showed no response a significant slowing in activation was observed in the Quin responding population before the pre-pulse in control conditions (see Figure 5.5B), which may suggest the presence of tonic G-protein modulation in this $\alpha_{1B}/rD2_L$ sub-population, possibly due to endogenous $\beta$ subunits. This is explained in further detail later (section 5.2.2.2).

The $\alpha_{1B}/rD2_L$ cell calcium channel current displayed inhibition by $\omega$-CgTx GVIA

An example $\alpha_{1B}/rD2_L$ whole cell patch clamp current recording that displayed sensitivity to the N-type VDCC blocker $\omega$-CgTx GVIA (300 nM) is shown in Figure 5.2. The time course of peak currents elicited in the cell by membrane depolarisations to +30 mV shows that block by $\omega$-CgTx GVIA (92% inhibition) was rapid and displayed minimal recovery over a 20 min wash duration. The block was observed across a range of voltages and no additional endogenous currents were unmasked by the $\omega$-CgTx GVIA block (Figure 5.2B).
Figure 5.2 An example calcium channel current recording from an $\alpha_{1D}/rD2_L$ expressing COS-7 cell displaying inhibition by $\omega$-CgTx GVIA.

(A) Time course of peak currents (open circles) elicited by depolarising the cell membrane of an $\alpha_{1D}/rD2_L$ expressing COS-7 cell. Voltage steps were from $V_{\text{hold}} = -80$ mV to $V_t = +30$ mV at 15 s intervals [see panel (C)]. $\omega$-CgTx GVIA (300 nM) perfusion is denoted by a black horizontal bar. At the start of the recording during control extracellular perfusion (CTRL, depicted by a filled circle) and during $\omega$-CgTx GVIA inhibition (depicted by a filled square) IV relationship voltage pulse protocols were performed. (B) The peak IV relationships that were recorded are shown: CTRL IV (filled circles) and $\omega$-CgTx GVIA inhibited IV relationship (filled squares). The IV relationship voltage step protocol used was similar to that described in Figure 5.1B, differing in the voltage step range (from $-20$ mV to $+45$ mV) and incremental steps (5 mV increments). (C) Example calcium channel current traces taken at points 1 (CTRL) and 2 ($\omega$-CgTx GVIA) during the time course. The voltage pulse protocol used is shown above the example traces.
5.2.2 Effect of pre-pulse and quinpirole on the $\alpha_{1B}/rD2_L$ and $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing COS-7 cells

5.2.2.1 $\alpha_{1B}$ channels display greater $G_{\beta\gamma}$ G-protein modulation when co-expressed with the $\beta_{2a}$ auxiliary subunit

*Time course of $rD2_L$ activation by quinpirole*

An example time course of peak currents recorded in $\alpha_{1B}/rD2_L$ expressing cells is shown in Figure 5.3A. Pre-pulse facilitation is apparent in this example, though does not show an obvious increase in the degree of facilitation during Quin perfusion. Current activation was not slowed in control conditions nor by application of Quin (see example traces above the time course in Figure 5.3A, and also mean current traces in Figure 5.4A), though calcium channel current inhibition was observed during Quin perfusion. In contrast, the $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells (Figure 5.3C) showed typical G-protein modulation of currents in both control (tonic G-protein modulation) and during Quin perfusion (due to GPCR activated $G_{\beta\gamma}$ subunits). Additionally, in this example recording wash out of Quin application resulted in current facilitation and a decrease in the pre-pulse facilitation. The difference in effect of Quin perfusion on $\alpha_{1B}/rD2_L$ and $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells can be seen in the mean normalised time courses (Figures 5.3B and D respectively). The mean normalised calcium channel current inhibition by Quin in $\alpha_{1B}/rD2_L$ expressing cells was $24.5 \pm 9.7\%$ ($n = 12$) and significantly different to control values ($p < 0.05$). In $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells the inhibition was $56.5 \pm 7.8\%$ ($n = 14$) and was significantly different when compared to both control and $\alpha_{1B}/rD2_L$ inhibited values ($p < 0.05$). The response to Quin in $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells was consistently observed (13/14 cells displayed calcium channel current inhibition) whilst in $\alpha_{1B}/rD2_L$ expressing cells Quin inhibition was inconsistent (only 6/12 cells displayed modulation).
Figure 5.3 G-protein modulation of α<sub>1B</sub> channels by rD2<sub>L</sub> receptor activation: (1) time course of action.

(A) and (C) show example time courses calcium channel currents recorded in α<sub>1B</sub>/rD2<sub>L</sub> and α<sub>1B</sub>/β<sub>2a</sub>/rD2<sub>L</sub> expressing cells respectively. The voltage pulse protocol used to elicit these calcium channel currents was as follows: V<sub>hold</sub> = -80 mV with a voltage step V<sub>t</sub> = +10 mV for 50 ms; this was followed 15 s later by the same V<sub>t</sub> = +10 mV preceded 10.2 ms before by a +120 mV pre-pulse of 100 ms duration. These voltage pulse protocols were performed cyclically, eliciting an I<sub>Ba</sub> without a pre-pulse and with a pre-pulse alternately, throughout the time course recordings. Example traces for control, 300 nM quinpirole (Quin) and wash elicited by such a pulse protocol are shown above the time course. The scale bar beside the first example trace (far left) applies to all the example traces. The voltage pulse protocol is provided above the control example traces [far left (A)], and it should be noted that the trace with a +120 mV pre-pulse (second from left) has been clipped to allow better trace resolution. The example time course for an α<sub>1B</sub>/rD2<sub>L</sub> expressing cell that displayed Quin I<sub>Ba</sub> inhibition (A) and for an α<sub>1B</sub>/β<sub>2a</sub>/rD2<sub>L</sub> expressing cell (C) depict I<sub>Ba</sub> measured at 20 ms into the test pulse, with I<sub>Ba</sub> without the +120mV pre-pulse depicted by square symbols, and I<sub>Ba</sub> with the +120 mV pre-pulse depicted by circles. In addition control and wash I<sub>Ba</sub> are depicted as open symbols, and I<sub>Ba</sub> recorded during Quin perfusion are depicted as filled symbols. The duration of the Quin perfusion is shown by the hatched shaded area. During the α<sub>1B</sub>/rD2<sub>L</sub> expressing cell recording (A) the time-course measurements were interrupted to perform a pre-pulse IV relationship (see Figure 5.4). The example recording from the α<sub>1B</sub>/β<sub>2a</sub>/rD2<sub>L</sub> expressing cell (C) also shows additional wash recordings and a second Quin application (far right of time-course). (B) and (D) show the mean normalised amplitudes for α<sub>1B</sub>/rD2<sub>L</sub> and α<sub>1B</sub>/β<sub>2a</sub>/rD2<sub>L</sub> expressing cells in response to Quin from recordings elicited and measured according to the pulse protocol described for the examples in (A) and (C). The same symbols and shading used in (A) and (C) also apply. In each experiment the currents were aligned to the point of the Quin application and normalised to the value 45 s (3 voltage pulse episodes) before the application of Quin. The mean inhibition of I<sub>Ba</sub> without pre-pulses (squares) was calculated to be 24.5 ± 9.7 % (n = 12) and 56.5 ± 7.8 % (n = 14) for α<sub>1B</sub>/rD2<sub>L</sub> and α<sub>1B</sub>/β<sub>2a</sub>/rD2<sub>L</sub> expressing cells respectively.
Further differences in the response to Quin in the two different channel compositions (with and without $\beta_{2a}$) were observed in IV plots recorded with (+PP) and without (noPP) +120 mV pre-pulses. The voltage pulse protocol to performed to investigate these +PP IVs and noPP IVs is shown in Figure 5.4A. Figures 5.4A and 5.4C give mean normalised current traces from a single voltage (+20 mV) from the IV families. Again, the $\alpha_{1b}/\beta_{2a}/\tau D_{2L}$ expressing cells ($n = 9$, Figure 5.4C) display distinct G-protein modulated current characteristics, notably: slowed current activation and subsequent removal by the +120 mV pre-pulse; pre-pulse facilitation in control and during Quin perfusion; and Quin induced current inhibition. These G-protein modulated current characteristics are not present in the mean normalised current trace for $\alpha_{1b}/\tau D_{2L}$ expressing cells ($n = 9$, Figure 5.4A). The IV relationships with and without the ±120 mV pre-pulse display a similar pattern of G-protein modulation present in the $\alpha_{1b}/\beta_{2a}/\tau D_{2L}$ expressing cells ($n = 9$, Figure 5.4D) and an absence of G-protein modulation in the $\alpha_{1b}/\tau D_{2L}$ expressing cells ($n = 9$, Figure 5.4C). Pre-pulse facilitation was observed across a range of voltages in both control and during Quin perfusion in the calcium channel currents recorded in $\alpha_{1b}/\beta_{2a}/\tau D_{2L}$ expressing cells. The channel modulation by G-proteins in these cells (with $\beta_{2a}$) was also emphasised by the significant difference ($p < 0.05$) in shifts of the $V_{50(Act)}$ and change in $k$ values calculated by modified Boltzmann fits to each of the IV relationships under differing conditions revealing G-protein modulation (the effect of Quin, pre-pulse effect in control and Quin perfusion conditions, summarised in Table 5.1). In contrast channels formed by expression of $\alpha_{1b}$ alone did not exhibit significant pre-pulse effects across the IV relationship voltage step range in either control or Quin G-protein activating conditions (Figure 5.4B). Indicative shifts of $V_{50(Act)}$ and changes in $k$ values were also not significant in response to Quin perfusion (Table 5.1). The $\alpha_{1b}/\tau D_{2L}$ expressing cells did, however, display a significant hyperpolarising shift (-3.3 mV, $p < 0.05$) and decreased voltage sensitivity (increased $k$ value, 125.4 %, $p < 0.05$) in the +PP IV compared to noPP IV in control conditions (Table 5.1).
Figure 5.4 G-protein modulation of $\alpha_{1B}$ channels by rD2L receptor activation: (2) the affect of pre-pulses on IV relationships in response to quinpirole.

(A) The voltage pulse protocol shown above the example trace (far left) was used to determine the effect of pre-pulses on IV relationships in control (CTRL) and quinpirole (+Quin) perfused conditions. A family of $I_{Ba}$ were elicited prior to a +120 mV pre-pulse (noPP currents) by stepping the cell membrane from $V_{\text{hold}} = -80$ mV to -40 mV and up to a final test pulse of +80 mV in 10 mV increment steps; each voltage step lasted 50 ms. The membrane was then returned to $V_{\text{hold}} = -80$ mV for 10 ms before the +120 mV pre-pulse was performed (with a duration of 100 ms). Another brief (10 ms) return to $V_{\text{hold}} = -80$ mV followed, and then the second set of $I_{Ba}$ traces were elicited by an IV exactly as for the noPP IV family, providing an IV family of $I_{Ba}$ with a pre-pulse (+PP IV family). Below this voltage step protocol is the mean normalised traces for calcium channel currents elicited by the voltage step protocol at the +20 mV voltage step in $\alpha_{1B}/rD2_L$ expressing cells in CTRL (denoted by trace with open symbols) and during Quin perfusion (denoted by trace with filled symbols). The symbols on the traces give the mean ± sem of the $I_{Ba}$ measured at these points (20 ms into the voltage step) for each of the conditions ($n = 9$). The scale bars represent one normalised current unit and 50 ms and apply to the example traces shown in (C). (B) The mean normalised IV relationships for noPP IV families (squares) and +PP IV families (circles) in CTRL (open symbols) and during Quin perfusion (filled symbols) in recordings made from $\alpha_{1B}/rD2_L$ expressing cells ($n = 9$). The IV families were elicited by the voltage pulse protocol described in (A). Elicited current amplitudes were normalised to the peak noPP $I_{Ba}$ in CTRL extracellular. The modified Boltzmann fit values (Equation 1, see Methods, section 2.7.5) that were applied gave the following parameter values: noPP CTRL conditions, $V_{50(\text{act})} = 15.3 \pm 2.4$ mV, $k = 5.7 \pm 0.4$ mV; +PP CTRL conditions, $V_{50(\text{act})} = 12.1 \pm 2.4$ mV, $k = 7.1 \pm 0.6$ mV; noPP with Quin, $V_{50(\text{act})} = 18.7 \pm 2.1$ mV, $k = 6.9 \pm 0.8$; and, +PP with Quin, $V_{50(\text{act})} = 16.0 \pm 3.1$ mV, $k = 7.8 \pm 1.0$ mV. The vertical arrow highlights the inhibition observed at the +20 mV voltage step. (C) The same as for (A) except recordings were made from $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells ($n = 9$). (D) The same as for (B) except recordings were made from $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells ($n = 9$). The modified Boltzmann fit values were as follows: noPP CTRL conditions, $V_{50(\text{act})} = 12.9 \pm 3.5$ mV, $k = 4.7 \pm 0.1$ mV; +PP CTRL conditions, $V_{50(\text{act})} = 9.6 \pm 3.4$ mV, $k = 3.6 \pm 0.2$ mV; noPP with Quin, $V_{50(\text{act})} = 24.8 \pm 4.2$ mV, $k = 7.4 \pm 0.4$; and, +PP with Quin, $V_{50(\text{act})} = 10.1 \pm 3.9$ mV, $k = 3.9 \pm 0.2$ mV. The changes in $V_{50(\text{act})}$ and $k$ in response to pre-pulses and Quin are summarised in Table 5.1.
Table 5.1 Summary of the effects of quinpirole and pre-pulse upon the IV relationship parameters $V_{50(\text{act})}$ and $k$ from currents recorded in $\alpha_{1B}/\beta_2 D_2L$ and $\alpha_{1B}/\beta_2 D_2L$ expressing cells.

IV relationships in $\alpha_{1B}/\beta_2 D_2L$ and $\alpha_{1B}/\beta_2 D_2L$ expressing cells (see Figure 5.4) were fitted with a modified Boltzmann equation (Equation 1, see Methods, section 2.7.5) before a +120 mV pre-pulse (noPP) and following a pre-pulse (+PP) in control conditions (CTRL) and during quinpirole (+Quin) perfusion. $V_{50(\text{act})}$ shifts were calculated by subtraction and changes in $k$ were displayed as percentages. An increase in $k$ indicates shallower voltage dependence, and steeper voltage dependence is indicated by decreased $k$ values. Where changes in $V_{50(\text{act})}$ and $k$ were significantly different they are denoted by * ($p<0.05$). Changes and significant differences were calculated between IV relationships in conditions defined in the far left column (e.g. the first row is for changes between IV relationships performed with noPP in CTRL and following Quin perfusion). The data analysed to give these figures were shown in the figure legend for Figure 5.4.

<table>
<thead>
<tr>
<th></th>
<th>$\alpha_{1B}/\beta_2 D_2L$ ($n = 9$)</th>
<th>$\alpha_{1B}/\beta_2 D_2L$ ($n = 9$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{50(\text{act})}$ shift (mV)</td>
<td>Change in $k$ (%)</td>
</tr>
<tr>
<td><strong>Quin Effect</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(noPP,CTRL) vs (noPP,+Quin)</td>
<td>$+3.3 \pm 1.8$</td>
<td>$121.0 \pm 10.6$</td>
</tr>
<tr>
<td><strong>Pre-pulse Effect (CTRL)</strong></td>
<td>$-3.3 \pm 0.5^*$</td>
<td>$125.4 \pm 9.4^*$</td>
</tr>
<tr>
<td>(noPP,CTRL) vs (+PP,CTRL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Pre-pulse Effect (+Quin)</strong></td>
<td>$-2.6 \pm 2.1$</td>
<td>$112.6 \pm 8.6$</td>
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5.2.2.2 Endogenous β subunit expression in COS-7 cells may account for the heterogeneity of quinpirole response in α1β/τD2L expressing cells

The sub-population of α1β/τD2L expressing cells that exhibit quinpirole sensitivity display altered pre-pulse characteristics

As was mentioned earlier (section 5.2.2.1) the response of calcium channel currents to Quin in the α1β/τD2L expressing cells was inconsistent: only 6/12 cells displayed Quin induced inhibition of the recorded calcium channel currents. To further analyse this disparity in response to Quin the α1β/τD2L expressing cells were sub-divided into two populations dependent on calcium channel current modulation by Quin. The results of analysing the pre-pulse facilitation ratio (+PP/noPP ratio) and the time to reach 90% of peak current (ttP90%) for the two α1β/τD2L cell populations (non-Quin inhibited and Quin inhibited) and the α1β/β2/τD2L expressing cell population are shown in Figure 5.5. As would be predicted from the previous data shown, the α1β/β2/τD2L expressing cells displayed significant differences (p <0.01) in all the comparisons highlighting current G-protein modulation. For example, significant differences were observed in +PP/noPP ratio between Quin and control conditions; noPP currents displayed greater ttP90% (slower activation) compared to +PP currents; and, the noPP currents during Quin perfusion displayed significantly greater ttP90% values compared to the ttP90% values observed in control conditions. The two α1β/τD2L expressing cell populations generally did not appear significantly different from each other. However, some factors were observed to be significantly different: the noPP currents recorded in control conditions for α1β/β2/τD2L cells displayed significantly slower (greater ttP90%) current activation kinetics than the same measurements performed in α1β/τD2L cells. In addition, in control conditions for each of the measurements (+PP/noPP ratio, noPP current ttP90% and +PP current ttP90%) the values calculated for α1β/β2/τD2L cells were significantly different from the corresponding values for the non-Quin inhibited α1β/τD2L.
Figure 5.5 Pre-pulse effects evaluated in $\alpha_{1B}/rD2_L$ (no Quin response), $\alpha_{1B}/rD2_L$ (Quin response) and $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells.

The following colour coding applies to both bar graphs: grey shading indicates recordings in control (CTRL) extracellular and black indicates recordings during quinpirole (Quin) perfusion. (A) The ratio of calcium channel current amplitude in noPP and +PP $I_{Ba}$ (+PP/no PP ratio) were determined for $\alpha_{1B}/rD2_L$, expressing cells that exhibited no Quinpirole (Quin) inhibition ($n = 5$, first two columns), for $\alpha_{1B}/rD2_L$ expressing cells that displayed obvious Quin inhibition ($n = 4$, third and fourth columns) and in $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells (columns five and six). A significant difference was observed between the ratio determined for $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells in CTRL and Quin conditions ($p < 0.01, **$). The scale is broken for greater clarity. The dotted line at +PP/noPP ratio = 1 highlights pre-pulse facilitation. Measurements were made from pre-pulse IV families as described in Figure 5.4A; the voltage step at which the measurements were made was at a voltage 10 mV hyperpolarised to the voltage step which elicited the peak calcium channel current amplitude, routinely +20 and +10 mV for $\alpha_{1B}/rD2_L$ and $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cell respectively. (B) Using the same recordings for the +PP/noPP ratio determined in (A) the time to reach 90 % of peak amplitude ($tp_{90\%}$) was also calculated for each of the cell populations. NoPP $I_{Ba}$ are indicated by the − symbol, whilst +PP $I_{Ba}$ are indicated by the + symbol below each column. Statistical significance is indicated by ** (p<0.01). Additional statistical significance (p<0.01) was also calculated between each of the CTRL measurements in the non-responding population of $\alpha_{1B}/rD2_L$ expressing cells (+PP/noPP ratio, noPP $tp_{90\%}$ and +PP $tp_{90\%}$) and $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells, but not between these cell populations and the Quin responding $\alpha_{1B}/rD2_L$ expressing cells; these significant differences are not indicated on the Figures (A) and (B) to aid clarity.
cells (p< 0.01), but non-significant when compared to the values for the Quin inhibited α1b/βD2L cells. These significant differences are not displayed in Figure 5.5 to aid clarity.

COS-7 cells have endogenous β3 mRNA

One explanation for the heterogeneous response observed in α1b/βD2L expressing cells during Quin perfusion was the contamination of pure α1b alone channels by endogenously expressed COS-7 VDCC β subunits. RT-PCR was performed on a mRNA extracted from a population of COS-7 cells with primer pairs to α1 (A-E, G), β (1-4) and α2δ VDCC subunits. Amplification products of the correct base pair sizes were detected by gel electrophoresis for α1E, β3 and α2 δ VDCC subunits (Figure 5.6).

Figure 5.6 Gel electrophoresis of RT-PCR products for VDCC subunits in COS-7 cells.

A gel showing the RT-PCR products amplified from a population of untransfected COS-7 cells. Correct sized bands are indicated by a red tick at the top right corner of the specified band. The amplification of the following subunits was performed: α1 A-E, G and β 1-4. To allow the determination of each of the amplified bands a 100 base pair (far left gel lane) and λ BstEII digest (far right gel lane) marker ladders were additionally electrophoresed.
5.3 DISCUSSION

5.3.1 Co-expression of β2a with α1B VDCC subunits – biophysical changes

The co-expression of VDCC β subunits with the pore forming α1 subunit has been previously shown to increase the current amplitude and produce a hyperpolarising shift in the current activation compared to α1 expression alone (reviewed by Walker and De Waard, 1998; Wakamori et al., 1999). A factor pertinent in this increase in amplitude of α1 currents by β subunit co-expression has been shown to be the translocation of α1 subunits to the cell plasma membrane by β subunits (Chin et al., 1995; Brice et al., 1997).

In these experiments typical IV relationships displaying hyperpolarised activation and increased current amplitude were observed when comparing current recordings from α1β/β2a/rD2L with α1β/β2a/rD2L expressing cells (Figure 5.1A), though both effects were non-significant between the two transfected cell populations. The hyperpolarising shift of approximately 10 mV was also observed in experiments performed independently by another member of Prof. Dolphin’s lab (Stephens et al., 2000) though in these experiments the αδ-1 was also co-expressed with the α1B and β2a VDCC subunits. Stephens et al. (2000) also observed Iba current densities approximately twice the size of the Iba densities observed in this study with α1β/β2a/rD2L expressing cells. This was most likely explained by either the variance in the current amplitudes recorded (30 ± 7 pA.pF⁻¹, n = 12; compared to 14.5 ± 7.9 pA.pF⁻¹, n = 8 observed in this study) or by the co-expression of αδ-1 in the study by Stephens et al. (2000). The co-expression of the αδ VDCC subunit has been shown to facilitate the effects of β subunit co-expression (including current amplitude increases, Klugbauer et al., 1999; Wakamori et al., 1999). Calcium channel currents in α1β/β2a/rD2L expressing cells also displayed slower current activation and a retardation of the rate of current inactivation compared to currents recorded in α1β/rD2L expressing cells (Figure
5.1B). These features were also characterised by Stephens et al. (2000). The similarity of these channel biophysical characteristics with previous studies provides confidence in the channel characteristics being unaltered by the additional co-expression of the rD2L receptor. In addition, the sensitivity of calcium channel current in α1β/rD2L cells to ω-CgTx GVIA (Figure 5.2), though not thoroughly investigated, also suggests that typical N-type calcium channel current characteristics are present in these transfected cells.

5.3.2 β2a co-expression modulates Gβγ G-protein inhibition

The unexpected conclusion from these experiments is that co-expression of the auxiliary β subunit enhances the voltage dependent Gβγ G-protein inhibition of calcium channel current through the α1 pore-forming VDCC subunit. The whole cell patch clamp recordings described here confirm convincing evidence from single channel and additional whole cell recordings made in Prof. Dolphin’s lab (Meir et al., 2000).

Calcium channel currents recorded in α1β/β2a/rD2L expressing cells displayed significant and characteristic G-protein modulation with tonic levels of Gβγ (control conditions) and enhanced modulation with increased free Gβγ concentration resulting from activation of the rD2L receptor by Quin perfusion. Pre-pulse facilitation, slowed current activation and IV relationship alterations all common to Gβγ G-protein modulated currents were all distinctly apparent in this population of α1β/β2a/rD2L expressing cells (Figures 5.3C, D, 5.4C, D, 5.5A, B and Table 5.1). In contrast the currents measured in the α1β/rD2L expressing cells displayed a reduced inhibition by Quin perfusion (Figure 5.3B). In these α1β/rD2L cells, other characteristics associated with the expected G-protein modulation arising from Quin perfusion, such as slowed current activation and enhanced pre-pulse facilitation, were not observed (Figure 5.3B, Figures 5.4A, B and Figures 5.5A, B).

Therefore, the lack or reduced levels (due to possible endogenous expression, see section 5.3.3, below) of β subunit, results in an apparently different G-protein modulation of calcium channel currents, since many of the typical characteristics are lost without the
presence of β subunits. It is possible that two or more pathways act concomitantly during usual Gpγ G-protein modulation of calcium channel currents: a minimal pathway that modulates currents in the presence only α₁β channels and a second additive pathway that is available in the presence of β subunits. The second pathway may explain the increased channel modulation observed with β subunits and account for the other features typically associated with G-protein modulation. Alternatively, a threshold level of β subunits (or specific β subunit isoforms) may need to be present to elicit the full range of G-protein modulation and associated traits. A mechanism that may account for the observed effects of β subunit co-expression, is that the presence of β subunits promotes the unbinding of Gβγ from the α₁ VDCC subunit (described in more detail in section 5.3.4).

5.3.3 Endogenous β subunit expression in COS-7 cells?

An explanation for the inconsistent Quin inhibition of calcium channel currents (6 cells displayed inhibition, 6 cells showed no effect) recorded in α₁β/rD₂L expressing cells is the presence of endogenous β subunits in the COS-7 cell line. It is possible that the responding cells had sufficient β subunit to elicit a G-protein response, whilst non-responding cells may have no or insufficient β subunit expression.

Analysis of pre-pulse effects (+PP/noPP ratio and ttp₉₀%) in α₁β/rD₂L Quin responding cells and non Quin responding α₁β/rD₂L expressing cells did provide indications that biophysical differences consistent with β subunit co-expression were apparent (Figure 5.5). The ttp₉₀% value for Quin inhibited α₁β/rD₂L cells was significantly greater than the ttp₉₀% value for Quin non-responding α₁β/rD₂L cells in control conditions. This significant slowed current activation was a characteristic of β₂a co-expression with α₁β channels in this study and in the study by Stephens et al. (2000). Further to this indication of endogenous β subunits altering properties in the Quin responding α₁β/rD₂L cells, all the control values measured in Figure 5.5 for the α₁β/β₂a/rD₂L expressing cells were significantly different (p<
0.01) from the control measurements in the non Quin responding $\alpha_{1B}/rD2_L$ cells. No
significant difference was determined when these values were compared between
$\alpha_{1B}/\beta_{2a}/rD2_L$ and the Quin responding $\alpha_{1B}/rD2_L$ expressing cells.

Evidence for the presence of endogenous $\beta$ subunit mRNA in COS-7 cells was also
provided by the RT-PCR of VDCC subunits in COS-7 cells (Figure 5.6). The amplification
of the $\beta_3$ VDCC subunit (as well as $\alpha_{1E}$ and $\alpha_{2\delta}$ subunits) mRNA adds weight to the
endogenous $\beta$ subunit explanation for the heterogeneous results observed in the $\alpha_{1B}/rD2_L$
expressing cells. Additional investigation using RT-PCR in Prof. Dolphin’s lab found the
endogenous presence of mRNA specific to four $\beta$ VDCC subunits ($\beta_{1b}, \beta_{2b}, \beta_3$ and $\beta_4$) in
COS-7 cells (Meir et al., 2000) – further evidence suggesting that $\beta$ subunits may in fact be
present and confusing the results observed in supposedly $\alpha_{1B}$ (alone) channels.

However, there are a number of limitations of the endogenous $\beta$ subunit explanation
for the variable Quin response in the $\alpha_{1B}/rD2_L$ expressing cells that also require highlighting.
The sub-division of the $\alpha_{1B}/rD2_L$ expressing cells into cells that showed Quin inhibition and
those that did not, displayed differences in some biophysical characteristics (e.g. ttp90%) 
suggestive of the presence of endogenous $\beta$ subunits in the Quin responding population.
Other characteristics that would also be expected to alter in the presence of $\beta$ subunits, such
as increased current amplitude and a hyperpolarising shift in the IV relationship, did not
exhibit a significant difference between the two $\alpha_{1B}/rD2_L$ expressing cell populations (data
not shown). However, though the difference in amplitude and IV shift was striking when
comparing IV relationships of currents recorded in $\alpha_{1B}/rD2_L$ (all cells, without sub-divisions
according to Quin response) and $\alpha_{1B}/\beta_{2a}/rD2_L$ expressing cells (Figure 5.1A), no significant
difference was detected for these traits between the cell populations. The RT-PCR results
must also be interpreted with care: as previously alluded to when discussing the RT-PCR
results for the GH4C1 D2 and HEK 293 $\alpha_{1D}$ cell lines, the presence of VDCC subunit mRNA
does not definitively clarify the presence of functional VDCC protein. In fact,
immunocytofluorescence performed within Prof. Dolphin’s lab suggested that although $\beta$
subunit VDCC mRNA was present in COS-7 cells the VDCC subunit protein was not (Meir et al., 2000). Thus, the endogenous β explanation requires further investigation to be fully acceptable as the reasoning for the disparate response to Quin in the α1B/β2L expressing cells.

5.3.4 Reconciling the unexpected β2a enhancement of G-protein modulation

Several previous studies have shown that co-expression of β subunits reduces calcium channel current inhibition by GPCR activated Gβγ pathways (Roche et al., 1995; Bourinet et al., 1996; Qin et al., 1997; Roche & Treistman, 1998). In Prof. Dolphin's laboratory, too, co-expression of β subunits reduced Gβγ G-protein inhibition of VDCC expressed in oocytes (Canti et al., 1999), and in experiments where the β subunit population of sensory neurons was depleted by anti-sense oligonucleotides, an increase in VDCC inhibition was observed during GABA_b receptor activation (Campbell et al., 1995a). In addition to these studies investigating the β subunit effect on G-protein inhibition of VDCC, protein binding studies have shown that the binding site on the α1 VDCC subunit for the β VDCC subunit and for the Gβγ G-protein dimers overlap (Zamponi et al., 1997; De Waard et al., 1997). These observations provided evidence for the theory of the mechanistic action of Gβγ inhibition of VDCC. The theory proposed that Gβγ dimers bind to the α1 VDCC subunit leading to inhibition of the calcium channel current. Since the Gβγ binding site on the α1 subunit is shared with the VDCC β subunit, the presence of β VDCC subunits may be expected to compete with the Gβγ dimers, and therefore reduce the inhibitory effect of the Gβγ dimers. Thus the increased G-protein modulation of the α1B VDCC by β2a co-expression described here appears awry with most of the present research and the consensus opinion of Gβγ inhibition of VDCC.

There are, however, some possible explanations that may account for the different modulation by β subunit co-expression. The majority of research cited for the reduction of
G-protein modulation by β subunit presence was performed in the *Xenopus* oocyte expression system. Although oocytes provide a very practical expression system, there are a wide range of endogenous proteins, including a functionally expressed β3 VDCC subunit (Tareilus *et al.*, 1997), which may confuse the activity of exogenous protein expression. The reduction in G-protein modulating effect that has been observed in oocytes may actually be arising due to competition between the endogenous β3 subunit and exogenous β subunit expression. Thus, the effects observed in oocytes may actually be a balance between modulation of channels formed of free α1, α1/endogenous β3 and α1/exogenous β VDCC subunits rather than the desired comparison between α1 channels with and without a defined exogenous β subunit. For example, in experiments performed in SCG neurons gene targeting was used to compare wild type and β3 subunit deleted SCG neuron VDCCs (Smith *et al.*, 1999). No difference in sensitivity to G-protein activating norepinephrine was observed between the two neuron populations, and an explanation suggested by the authors was the confounding effect of endogenous β1 and β4 subunits present in SCG neurons. Since endogenous β subunit may also be present in COS-7 cells (see section 5.3.3) the confusing interaction between endogenous/exogenous β subunits may also be applicable to the β2α co-expression effect described here. However, a similar enhancement of G-protein modulation was also observed with β1b co-expression in COS-7 cells, which argues against specific competition by isoforms of β subunits. Another factor that may account for the difference between β subunit effects observed here and in other research, is that many of the previous studies have quoted isopotential inhibitions. The inhibition calculated with β subunit co-expression will therefore inherently be affected by the hyperpolarising shift in the current activation induced by β subunits, making clear comparisons difficult.

The antisense depletion of β subunits by Campbell *et al.* (1995b) in sensory neurons also provided data showing that β subunits antagonise the G-protein modulation effect on VDCC currents, in opposition to the enhancement effect shown here. In discussing this, Meir *et al.* (2000) provided several explanations for the disparity in results. For example, the
voltage dependent $G_{\beta Y}$ G-protein modulation investigated here is only one pathway that may be activated in native neurons. Additional pathways found in native neurons, including voltage independent modulation of VDCC currents have been shown to be activated by G-protein activation (Diverse-Pierluissi et al., 1991; Fitzgerald & Dolphin, 1997; Currie & Fox, 1997; Smith et al., 1999). When additional factors such as cross-talk between $G_{\beta Y}$ voltage dependent and voltage independent pathways (Zamponi et al., 1997), and the modulatory effects of the $G_{\alpha}$ subunit are considered (Kammermeier & Ikeda, 1999), it becomes apparent that there may be sufficient complexity of G-protein modulation of VDCC currents to allow differing effects to be observed in the various studies performed. Thus, the different expression systems and different combinations or levels of $\alpha_1$ and $\beta$ subunits may make the opposing observations of the enhanced effect of $\beta_{2a}$ co-expression described here and the present research on $\beta$ subunit more comprehensible taken within the context of the specific recording conditions.

The overlapping binding sites on the $\alpha_1$ VDCC subunit for the $\beta$ VDCC subunit and the $G_{\beta Y}$ suggest a mechanism in which there is competition between the $\beta$ subunit and the $G_{\beta Y}$ for the binding site, and hence leads to the presently accepted proposal of the $\beta$ subunit antagonising the $G_{\beta Y}$ modulating effect upon VDCC currents. In the paper that includes the experiments described here, Meir et al. (2000) suggest that the overlapping binding site mechanism may still be applied to the enhanced G-protein modulation by $\beta_{2a}$ co-expression. In applying the theory of overlapping binding $\beta$ VDCC subunit/$G_{\beta Y}$ sites it was suggested that the presence of the $\beta$ subunit would increase the $G_{\beta Y}$ unbinding from the $\alpha_1$ subunit due to pre-pulse depolarisation. Thus, the VDCC $\beta$ subunit effect may be described as an antagonistic effect to the $G_{\beta Y}$ binding, and directly affecting the rate of $G_{\beta Y}$ unbinding from the VDCC $\alpha_1$ subunit. The $G_{\beta Y}$ subunit binding to the VDCC $\alpha_1$ subunit causes the voltage-dependent effects associated with G-protein modulation, and therefore $\alpha_{1B}$ channels (with no $\beta$ subunit) may be largely lacking in the voltage-dependent activity exhibited by $G_{\beta Y}$.
subunits. Hence, the minimal inhibition and distinct modulation observed here without \( \beta \)
subunit co-expression, may suggest an alternative pathway is active or unmasked in \( \beta \)
subunit absence (see section 5.3.2), or that endogenous \( \beta \) subunits are present in the cells that
responded in the no exogenous \( \beta \) subunit experiments (see section 5.3.3). Using this
argument the oocyte experiments, and possibly the \( \beta \) subunit antisense experiments, cited
earlier may possibly be explained by assuming the low levels of \( \beta \) subunits present in these
experiments results in an altered \( G_{\beta} \) unbinding rate. This \( G_{\beta} \) unbinding rate would be
further altered in the presence of exogenous \( \beta \) subunit expression, and being a voltage-
dependent process the \( G_{\beta} \) unbinding rate would result in differing levels of inhibition (and
the corollary pre-pulse facilitation) at different voltage potentials that was shown here
(Figure 5.4D). Further evidence for this voltage-dependent \( G_{\beta} \) unbinding rate affecting
VDCC current inhibition was suggested by experiments in oocytes in which exogenous \( \beta_3 \)
co-expression with the \( \alpha_{1B} \) VDCC subunit was performed (Roche & Treistman, 1998). The
\( \beta_3 \) subunit was shown to decrease current inhibition at certain test potentials, test potentials
that maybe expected to exhibit increased \( G_{\beta} \) unbinding rate in the presence of VDCC \( \beta \)
subunits; in addition increased pre-pulse facilitation (or voltage-dependent relief of \( G_{\beta} \)
inhibition) was observed with \( \beta_3 \) co-expression (Roche & Treistman, 1998).
CHAPTER 6

CONCLUDING REMARKS
Summary

The principal aim of this study was to investigate the G-protein modulation of the neuroendocrine L-type calcium current. In this study it was shown that a neuroendocrine calcium current (in the GH4C1 D2 cells) and an expression system modelling the neuroendocrine L-type calcium channel complex (in the HEK 293 α1D cells) clearly exhibited L-type calcium current biophysics and pharmacology. However, in each case G-protein modulation of the calcium currents was not observed. Thus, further experimentation in this area is required to elucidate the molecular determinants of the neuroendocrine L-type calcium currents that have been shown to exhibit G-protein modulation.

The aspect of the interactions of the VDCC β subunit with the VDCC α1B subunit and Gβ, G-protein was also investigated. In particular, further investigation was performed in response to the single channel recordings performed by Meir et al. (2000), in which the co-expression of VDCC β subunits were shown to be necessary for the characteristic G-protein modulation of expressed α1B channels. This effect with VDCC β subunit co-expression was investigated in this study using the whole-cell patch clamp configuration. The results described here provide further evidence for the VDCC β subunit providing the requisite protein interactions to allow complete and characteristic G-protein modulation of the α1B channel current to occur.
Overview to Chapter 3

**GH4C1 D2 cells did not exhibit G-protein modulation**

In experiments described in Chapter 3 no G-protein modulation of the GH4C1 D2 cell line current was observed. Modulation was examined using endogenous (sst2 and M4) and exogenous (huD2s) GPCR pathways, directly by intracellular GTP-γS dialysis and by observation of pre-pulse facilitation (another characteristic G-protein inhibition).

Considering the substantial evidence for G-protein modulation of calcium channel currents in GH cells, including GH3 (Kleuss et al., 1991; Piros et al., 1995; Zong et al., 1995b), GH4C1 (Liu et al., 1994) and clonal GH4C1 D2 and GH4C1 D4 cells (Seabrook et al., 1994) these data presented here are difficult to explain.

*Possible explanations for the disparity in modulation between other neuroendocrine currents and the GH4C1 D2 data*

All the components used in the intracellular and extracellular solutions used here have been widely used in such studies previously before, and hence should allow G-protein modulation to be observed. The cell culture of the GH4C1 D2 cells was performed according to culture conditions (environment and solutions) described by Seabrook et al. (1994) for this cell line. In subsequent experiments striking G-protein modulation of α1B currents (known to be G-protein modulated) have been observed using GPCR and direct (GTP-γS) activation of G-proteins in HEK 293 (see Chapter 4, section 4.2.4.1) and COS-7 (see Chapter 5, section 5.2.2) cells. Therefore I am confident that I would have observed G-protein modulation of the current if it had existed.

An obvious and logical explanation for this loss of G-protein modulation of the GH4C1 D2 cell line currents is not easily formulated from the data presented. One difference in the recordings presented here compared to that of Seabrook et al. (1994)
was a change of the charge carrier used (Ba\(^{2+}\) rather than Ca\(^{2+}\), respectively). In a series of experiments Hille and colleagues observed a muscarinic agonist activated pathway that inhibited calcium currents in rat superior cervical ganglion neurons (Beech et al., 1991; Bernheim et al., 1991; Beech et al., 1992; Mathie et al., 1992). The current inhibition was shown to be calcium specific and was blocked by intracellular calcium chelation using BAPTA. Intracellular calcium was buffered by EGTA present in the experiments performed by Seabrook et al. (1994) and also in the experiments described here. Therefore, the presence of EGTA might be expected to prevent the possibility of the inhibitory pathway that was observed by Hille and co-workers. However, it may be speculated that when Seabrook and colleagues observed modulation in some GH\(_4\)C\(_1\) D2 cells, the modulation was observable due to imperfect intracellular Ca\(^{2+}\) chelation in these modulated cells, due to saturation by external Ca\(^{2+}\). This scenario would not arise in the experiments explained here, since the charge carrier was Ba\(^{2+}\). The calcium channel current recording in this study that was shown to be inhibited by Quin perfusion (see Figure 3.1) was performed using Ba\(^{2+}\) as the charge carrier, and this result therefore remains incongruous with respect to the hypothesis of a Ca\(^{2+}\)-specific pathway reasoning.

The only calcium channel current (though carried by Ba\(^{2+}\)) shown to exhibit G-protein modulation was recorded at the MSD laboratories. The data presented has suggested that a crucial factor in the G-protein/GPCR transduction pathway was missing or aberrant in the GH\(_4\)C\(_1\) D2 cell studied, though the nature of this pathway component was not discovered. The missing G-protein pathway protein or factor suggests an inherent problem in the cells, possibly through growth, storage and/or transport. Every effort was made to ensure cells were unmodified by the transport and subsequent growth of cells at UCL: cells were transported with dry-ice, preventing unnecessary freeze-thawing in transit; numerous aliquots were grown and investigated to ensure an aberrant aliquot of cells was not the cause of the loss of G-protein modulation. When growth was resumed at UCL, no difference in culture conditions between MSD and UCL was determined. However, despite all the careful treatment of the cells between the two sites, the
explanation of modification of the GH4C1 D2 cells due to aberrant cell growth provides the greatest number of possible variables and hence sources for error. Therefore, the cell culture may be the most likely source for the loss of G-protein activation in the GH4C1 D2 cell calcium channel current. This explanation is supported by the fact that in addition to the exogenous huD2s receptor exhibiting no response, the endogenous pathways coupling to the sst2 and M4 receptors were also insensitive in these cells, despite previous studies showing that G-protein modulation of the VDCC currents by these pathways was possible (see section 3.2.3.1).

Overview to Chapter 4

A neuronal L-type current model lacks modulation by G_{p Y}

Experiments in which G_{p Y}-mediated modulation of the \( \alpha_{1D} \) channel currents expressed in the HEK 293 \( \alpha_{1D} \) cells were performed. These experiments investigated whether \( \alpha_{1D}/\alpha_{5}\delta-1/\beta_{3a} \) channel currents provided the molecular VDCC composition that corresponded to neuroendocrine L-type currents that had been shown to exhibit G-protein modulation by several research groups. The investigations on HEK 293 \( \alpha_{1D} \) cells used endogenous (sst2) and transiently expressed (rD2l) GPCRs; activation by GTP-\( \gamma \)S; and also attempted to induce G-protein modulation by the second messenger pathways involving PKA phosphorylation. A range of protocols was used to examine G-protein modulation including: whole-cell and perforated-patch clamp, various depolarising pre-pulse protocols and measurements (ttp\% and +PP/noPP ratio). Across all these conditions and protocols no G-protein modulation was ever observed in the HEK 293 \( \alpha_{1D} \) (\( \alpha_{1D}/\alpha_{5}\delta-1/\beta_{3a} \) expressing) cells.

From these results, the \( \alpha_{1D} \) L-type channel does not appear to be the molecular counterpart of the native neuroendocrine L-type channel currents that have been shown to
exhibit G-protein modulation in several neuroendocrine preparations (Kleuss et al., 1991; Haws et al., 1993; Tallent et al., 1996; Gilon et al., 1997; Degtiar et al., 1997; Hernandez-Guijo et al., 1999). Further to the observations made here, results from an α1D knockout mouse have recently been published (Platzer et al., 2000). The α1D knockout mice did not appear to exhibit neurological dysfunction in relation to hormone and neuropeptide exocytosis, with the α1D knockout effect apparently being limited to auditory transduction pathways and pacemaker activity at the sinoatrial node. Thus, the α1D calcium channel appears to perform distinct roles from the G-protein modulated, neuroendocrine secretory function initially ascribed to this α1 isoform.

Source of G-protein modulated neuroendocrine L-type current?

Since this investigation has shown that the α1D/α2δ-1/β3 channels do not exhibit G-protein modulation, what is the molecular source of the L-type current in neuroendocrine cells that do exhibit G-protein modulation? Previous work in Prof. Dolphin's lab showed DHP antagonist block of another rat α1E isoform, rbEII (Stephens et al., 1997). However, this isoform has a 50 amino acid truncated N terminus (Page et al., 1998), and may not represent a native isoform in rat brain (Page et al., 1998; Schramm et al., 1999). In the research presented here further confirmation of this α1E DHP sensitivity result has been shown using α1Elong, an isoform whose extended N terminus is homologous to the cloned human (accession no.L27745), rabbit (X67855) and mouse (L29346, Williams et al., 1994) α1E sequences. The partial DHP sensitivity (particularly to nicardipine) of α1E currents shown here, as well as the DHP sensitivity of other cloned non-L type currents observed recently (Furukawa et al., 1999; Burley and Dolphin, 2000) suggests the caveat that that some studies apparently demonstrating G-protein modulation of 'L-type' currents (according to their sensitivity to DHP antagonists) may need to be reviewed. However, this confusion over DHP selectivity may only account for a few studies, and the significant bank of evidence for G-protein modulation of neuroendocrine L-type
channels will be unaffected, particularly those in which L-type currents have been defined by S(-)-BayK8644 enhancement (Hernandez-Guijo et al., 1999).

Additional α_{ID} isoforms have been cloned from pancreatic β-cells in rat (Ihara et al., 1995) and hamster (Yaney et al., 1992). There is little functional expression data available for these clones. Expression of α_{ID} clones appears to be problematic and is generally erratic (even in the clone used in this study a low percentage of cells exhibited stable currents) with relatively low current density yields, a problem that has hindered research in this area; and may indicate that the full-length clones currently available are not naturally occurring splice variants. A number of sequences within the α_{IA}, α_{IB} and α_{IE} VDCC subunit amino acid clones have been shown to be important for G_{βγ} binding and modulation of the channel. These important α_{I} VDCC subunit sequences include the intracellular linker between domains I and II (De Waard et al., 1997), a region within the N-terminus (Page et al., 1998; Canti et al., 1999); and the C-terminus (Zhang et al., 1996; Qin et al., 1997). Two particularly relevant sequences in the I-II linker (-QQIER-) and the N terminus (-YKQSLAQRART-) are not present in the α_{ID} cloned used here (see section 1.4.3 and Figure 1.6). When comparing sequence alignments of the pancreatic β-cell α_{ID} clones with the neuronal α_{ID} clone used in this study (not shown), most elements in regions that may be pertinent to G-protein modulation are homologous to each other. This suggests that these additional published α_{ID} clones may also be predicted to exhibit no G-protein modulation. Indeed, similar results regarding lack of inhibitory G protein modulation using another α_{ID} clone (Yaney et al., 1992) have been obtained (A. Scholze, T. D. Plant, A.C. Dolphin and B. Nürnberg, unpublished results). However, the sequence homologies show least conservation in the C-terminal sequences of these α_{ID} clones (a characteristic observed in all the α_{I} VDCC subunits), providing scope for the possibility that the C-terminus of alternative α_{ID} splice variants may provide the ability to be G-protein modulated. Clearly, the problems encountered concerning functional expression of the alternative α_{ID} clones will need to be overcome in order to answer these questions.
As further progress is made in the elucidation of neuroendocrine L-type channels it is becoming clear that a sophisticated level of complexity is likely to exist. For example, in the GH\textsubscript{3} (related to the GH\textsubscript{4}C\textsubscript{1}D\textsubscript{2} cells investigated in Chapter 3) cell line alone, several mRNA transcripts encoding splice variants of the $\alpha_{1D}$ subunit have been shown to exist (Safa \textit{et al}., 1998). Further variations of these channel complexes will be added due to the differing combinations of accessory subunits. Although $\beta_3$ appears to be a significant accessory subunit associated with neuronal L-type channels (Pichler \textit{et al}., 1997), nevertheless, $\beta_4$ is also prominently associated with neuronal L-type channels, and $\beta_{1b}$ and $\beta_{2a}$ are also present though in small proportions (Pichler \textit{et al}., 1997). Between $\beta$ subunit isoforms there are also splice variants (see Table 1.3; for reviews see Castellano and Perez-Reyes, 1994; Birnbaumer \textit{et al}., 1998), which add to the possible channel subunit complexes. Amongst these combinations of $\alpha_{1D}$ splice variants and accessory subunits there may be a sub-set that do exhibit the G-protein modulation observed in native neuroendocrine cells and derived cell lines. Alternatively, an as yet undiscovered accessory protein may be required for coupling of the neuronal L-type channels to G-protein inhibitory pathways (see further discussion, later).

**Overview to Chapter 5**

$G_\beta$ modulation of VDCC currents requires VDCC $\beta$ subunits

The effects of VDCC $\beta$ subunits on the $G_\beta$ modulation of $\alpha_{1B}$ VDCC currents were investigated using heterologous expression of $\alpha_{1B}$ channels with and without $\beta$ subunit coexpression. Previous research suggested that VDCC $\beta$ subunits reduced G-protein modulatory effects upon VDCCs (Roche \textit{et al}., 1995; Campbell \textit{et al}., 1995a; Bourinet \textit{et al}., 1996). Apparently in contrast to this previous research, the data described here suggests that $\beta$ subunit co-expression is necessary for the full G-protein modulation of the...
\( \alpha_{1B} \) VDCC current. Typical GPCR activated \( G_{\beta\gamma} \) modulation characteristics include: pre-pulse facilitation; slowed current activation kinetics; a depolarisation of the IV relationship; and inhibition of VDCC current amplitude. When these characteristics were investigated in channels composed of \( \alpha_{1B} \) alone, the response to Quin activation of the co-expressed rD2L receptor was inconsistent and only displayed a decreased current amplitude inhibition without the additional traits that are routinely observed with \( G_{\beta\gamma} \) modulation.

Mechanistic explanation for the \( \beta \) subunit effect observed in this study

The reduced inhibition and range of characteristics of G-protein modulation in the absence of \( \beta_2 \) co-expression was also observed in further experiments performed in Prof. Dolphin's lab, and was explained by \( G_{\beta\gamma} \) preventing access of the \( \beta \) subunit to the sites at which it modulates channels and an increased \( G_{\beta\gamma} \) unbinding rate at depolarised potentials in the presence of \( \beta \) subunit (Meir et al., 2000). The inconsistent and reduced modulation in the \( \alpha_{1B} \) (alone) channels may arise due to low levels and/or different \( \beta \) subunit expression endogenous to the COS-7 cells used. This \( G_{\beta\gamma} \) unbinding rate explanation, discussed more fully in section 5.3.4, may also reconcile some of the apparently opposing observations of \( \beta \) subunit effects on G-protein modulation made by several other groups.
Areas of further investigation highlighted by this study

In discussing the molecular derivation of the neuroendocrine L-type channel current that exhibits G-protein modulation (see above), it was suggested that subunit isoforms and/or combinations might provide the neuroendocrine L-type channel complex that is sensitive to G-protein modulation. Thus, on the G-protein sensitivity precedent alone, it may be suggested that there is still at least one L-type isoform yet to be discovered. The G-protein insensitive $\alpha_{1D}$ channel studied in this investigation obviously does not fulfil the requirements of the undefined L-type current. Further evidence supporting the notion of another undefined L-type isoform is also provided by the $\alpha_{1D}$ knockout mouse study cited earlier (Platzer et al., 2000), since if the $\alpha_{1D}$ channels were source of the G-protein sensitive neuroendocrine L-type current more extensive neuroendocrine disorders than the reported auditory disruption would be predicted. With \textit{in silico} techniques discovering putative channel subunits with increasingly less homology to the primary sequences of existing subunits (and hence their cloaking within the genome), though displaying strong homologies in secondary structural determinants, it is highly probable that several more VDCC subunits will be found. The putative L-type isoform $\alpha_{1F}$ (see Table 1.2) has yet to be functionally expressed. However, $\alpha_{1F}$ is an unlikely source for the modulated neuroendocrine L-type current, since $\alpha_{1F}$ appears to be exclusive to retinal tissue. The discovery of a new L-type clone may also be suggested by the difficulty of expression of known $\alpha_{1D}$ isoforms (see Introduction, section 1.3.3). An alternative explanation for the poor expression of these clones is that \textit{in vivo} $\alpha_{1D}$ undergoes additional post-translational processing (\textit{e.g.} proteolytic cleavage) that has yet to be replicated by \textit{in vitro} expression studies; this protein processing could also equally reveal G$\beta$7 binding/modulating sequences.
In addition to specific subunit combinations (including new L-type \( \alpha_1 \) isoforms) or post-translational modifications as a source for the undetermined molecular nature of the modulated neuroendocrine L-type current, a further explanation that was suggested earlier is the possibility of an undefined accessory protein that may be required for efficient G-protein coupling/transduction with the L-type channel. Though undefined, this protein may only be undefined in as much that the G-protein/L-type channel accessory activity has yet to be determined. Thus, existing protein interactions that have been shown to occur between several proteins and L-type channels (see section 1.4.5) would provide a useful starting point to investigate this possibility. Of the proteins already known to interact with \( \alpha_1 \) VDCC subunits, syntaxin probably offers the most likely source of an accessory protein to the mechanism of G-protein/L-type binding and modulation. Syntaxin has been shown to bind to \( \alpha_{1A} \) (Rettig et al., 1996), \( \alpha_{1B} \) (Sheng et al., 1994) and \( \alpha_{1D} \) subunits (Yang et al., 1999). The interaction of syntaxin has been shown to optimise the G\(_{\beta\gamma}\) binding to \( \alpha_{1B} \) channels (Jarvis et al., 2000). Therefore, it would be interesting to investigate whether a similar G\(_{\beta\gamma}\) binding optimisation would be possible with \( \alpha_{1D} \) channels coexpressed with syntaxin, a binding optimisation that may elicit G-protein modulation of the channels. Considering the G-protein modulation of the neuroendocrine L-type current has frequently been observed in endocrine or neurosecretory cells, the suggestion of the accessory protein being syntaxin, a key member of the exocytic protein complex, is an intriguing prospect.

Further investigation is also required to fully define the VDCC \( \beta \) subunit effect upon G-protein modulation of VDCC currents. The unexpected results observed by Meir et al. (2000) and subsequently investigated and discussed in Chapter 5, showed that \( \beta \) subunits are required for a complete range of G\(_{\beta\gamma}\) modulatory effects on the VDCC current. One criticism that may be levelled at this research is that investigations of this \( \beta \) subunit
effect, so far, are limited to the COS-7 cell line. COS-7 cells have provided a useful model for the study of heterologous VDCC expression and their modulation by G-proteins, though more studies in additional model cell lines would undoubtedly strengthen the β subunit requirement hypothesis suggested by the COS-7 cell studies. Considering the extensive molecular manipulation of protein expression in cells that is now available, for instance by gene-carrying plasmid overexpression or antisense oligonucleotide knockout methodology, investigation in native tissue slices or acutely isolated cells would be of particular interest, with the intention of providing further corroborating evidence using endogenous, native G-protein pathways. The necessity for performing the VDCC β subunit coexpression studies in a cell line that is shown to be entirely devoid of endogenous β subunits is also of paramount importance in definition of these protein interactions. Alternative paradigms that could attempt to achieve an expression system with no VDCC β subunit present, or at least decrease basal levels of VDCC β subunit, include using a conserved β subunit antisense oligonucleotide strategy (e.g., see Berrow et al., 1995; Campbell et al., 1995b) or using α1b I-II loop fragment overexpression. The latter suggestion, using α1b I-II loops, would not decrease VDCC β subunit levels, though they would be expected to act as ‘sinks’ to reduce VDCC β subunit functionality. However, this suggestion would be very challenging technically, since the I-II loops would also be likely to interact with the Gp subunits: the challenge would be to define fragments of the I-II loop that were exclusively interactive with the VDCC β subunit. A further variation on the investigations of different levels of VDCC β subunit and the resulting effect upon VDCC current modulation by Gp, would be the overexpression and subsequent flooding of the cell with mutant forms of the VDCC β subunit. Expressed VDCC β subunit mutants, particularly mutations within the β sequence that interacts with the α1 subunit (BID; see review by Walker and De Waard, 1998), would provide models in which varying levels of functional VDCC β subunit would be present. Such manipulation of the β subunit functionality by mutation would
suggest mechanistic interactions of the $\alpha_i/\beta/G_\beta\gamma$ complex and delineate roles of each protein within the complex, and hence the resulting VDCC current modulation.
Abstracts and Publications

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